The Role of PASKIN
in Metabolism and Protein Synthesis

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Philipp Schläfli

von Albligen BE

Promotionskomitee

Prof. Dr. Roland H. Wenger (Vorsitz und Leitung der Dissertation)
Prof. Dr. Carsten A. Wagner
Prof. Dr. Florian Lang
Dr. Daniel P. Stiehl

Zürich, 2011

This work has been performed under the supervision of

Prof. Dr. Roland H. Wenger

at the Institute of Physiology of the University of Zürich

within the PhD program in Integrative Molecular Medicine (ImMed)
of the Zürich Center for Integrative Human Physiology (ZIHP)

TABLE OF CONTENTS

1 Abbreviations 1

2 Zusammenfassung 5

3 Summary 9

4 Introduction 11

4.1 Per-ARNT-Sim (PAS) domains 11
4.2 Human PASKIN, a PAS domain containing Ser/Thr kinase 12
4.2.1 PASKIN gene structure 13
4.2.2 The PAS domain of PASKIN 13
4.2.3 The kinase domain of PASKIN 14
4.3 PASKIN in yeast 16
4.4 Energy homeostasis 17
4.4.1 PASKIN and glucose homeostasis 18
4.5 Protein synthesis 20
4.5.1 PASKIN function in protein translation 22
4.5.2 Eukaryotic elongation factor 1A1 23
4.5.3 Ribosomal protein S6 24
4.6 Other PASKIN functions: testis and regulation of ventilation 26
4.7 Phospholipids 26
4.7.1 Phosphatidylinositides 28
4.7.2 Phosphatidylinositide binding motifs 31
4.8 Goals of the thesis 35
4.9 References 36

5 Manuscript I: Review Article 49
"The PAS-domain kinase PASKIN: a new sensor in energy homeostasis"

6 Manuscript II: Original Article 57
"Male Germ Cell Expression of the PAS Domain Kinase PASKIN and its Novel Target Eukaryotic Translation Elongation Factor eEF1A1"

7 Manuscript III (Submitted) 71
"Substrate preference and phosphatidylinositol monophosphate inhibition of the catalytic domain of the PAS kinase PASKIN"

8 Manuscript IV (Unpublished) 93
"*Paskin*$^{-/-}$ mice are protected from detrimental effects of obesity"

9 Manuscript V (Unpublished) 119
"PASKIN is predominantly expressed in leukocytes and is localized along the actin cytoskeleton"

10 Conclusions 133

11 Curriculum vitae 143

12 Original publications and own contributions 145

13 Acknowledgements 147

1 ABBREVIATIONS

4E-BP	4E binding protein
aa-tRNA	amino-acylated-transfer-RNA
ADP	adenosine diphosphate
Aer	aerotaxis sensor receptor
AhR	aryl hydrocarbon receptor
AKT	RAC serine/threonine-protein kinase, Protein kinase B (PKB)
AMPK	AMP-activated kinase
AMP	adenosine monophosphate
ANTH	AP180 N-terminal homology
AP	adaptor/assembly protein
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
bHLH	basic helix– loop–helix DNA-binding domain
CAF20	Cap-associated factor 20
CoA	coenzyme A
CREB	cAMP response element binding protein
DAG	diacylglycerol
DOG	dioctanoylglycerol
EEA1	early endosome autoantigen 1
eEF	eukaryotic translation elongation factor
EGFP	enhanced green fluorescent protein
eIF	eukaryotic translation initiation factor
ENTH	epsin N-terminal homology
ERK	extracellular signal-regulated kinase
ESCRT	endosomal sorting complex required for transport
FAPP	Feto-Acinar Pancreatic Protein
FAS	fatty acid synthase
FMN	flavin mononucleotide
GLUT	glucose transporter
Gsy	glycogen synthase
GDP	guanosine diphosphate
GSK	glycogen synthase kinase
GST	glutathione-S-transferase
GTP	guanosine trisphosphate

HERG	human *eag*-related gene
HFD	high fat diet
HIF	hypoxia-inducible factor
INS	insulin
IP_3	inositol trisphosphate
IR	insulin receptor
IRS	insulin receptor substrate
Kif	kinesin family
LPS	lipopolysaccharides
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MIN6	mouse insulinoma cell line
mTOR(C)	mammalian target of rapamycin (complex)
mRNA	messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NCD	normal chow diet
NPAS	neuronal PAS protein
NMR	nuclear magnetic resonance
NOXO1	NADPH oxidase organizer 1
P	phosphate
PA	phosphatidic acid
PARP	Poly (ADP-ribose) polymerase
PAS	PER/ARNT/SIM
PC	phosphatidylcholine
PDK-1	phosphatidylinositol phosphate-dependent kinase-1
PDX-1	pancreatic duodenal homeobox-1 transcription factor-1
PE	phosphatidylethanolamine
PER	*Drosophila* period clock protein
PGC	Peroxisome proliferator-activated receptor gamma coactivator
PH	pleckstrin homology (domain)
PHOT	phototrophin
PI(P)	phosphatidylinositol (phosphate)
PI3K	phosphatidylinositol 3-kinase
PIKfyve	phosphoinositide kinase, FYVE finger containing
PKA	protein kinase A
PLC/D	phospholipase C/D
PKC	protein kinase C

PS	phosphatidylserine
PtdIns	phosphatidylinositol
PTEN	phosphatase and tensin homolog
PYP	photoactive yellow protein
RNA	ribonucleic acid
S	sphingomyelin
S6K	p70 S6 kinase
SIM	*Drosophila* single-minded protein
Sds22	protein phosphatase, regulatory subunit 7
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment receptor
Snf1	sucrose non-fermenting 1 protein kinase
TIAM1	T-cell lymphoma invasion and metastasis 1
Tif11	translation initiation factor 1A
Tirap	toll-interleukin 1 receptor domain containing adaptor protein
TOP	terminal oligopyrimidine
TSC	tuberous sclerosis complex
Ugp	UDP-glucose pyrophosphorylase
Vam7	vacuolar morphogenesis protein 7
VPS	vacuolar protein sorting

2 ZUSAMMENFASSUNG

PER-ARNT-SIM (PAS) Domänen sind in Organismen verbreitet. In Bakterien und Archaeen fungieren sie hauptsächlich als sensorische Module für eine Vielzahl von Umwelteinflüssen. Dies geschieht entweder direkt durch Binden des zu detektierenden Liganden oder indirekt unter Verwendung eines Kofaktors. Dabei induziert die Detektion Veränderungen in der Konformation der PAS-Domäne, welche das Signal zu einer Effektordomäne, häufig einer Kinase, weiterleitet. In Eukaryoten, insbesondere in basic helix-loop-helix Transkriptionsfaktoren, ermöglichen PAS-Domänen oftmals das Dimerisieren zweier Proteine. Das PASKIN-Protein besitzt die für Eukaryoten einzigartige Kombination einer PAS-Domäne und einer Serin/Threonin Kinase und ist von der Hefe bis zum Menschen konserviert. Für die PAS-Domäne von PASKIN wurde ein Modell propagiert, gemäss welchem ein unbekannter metabolischer Ligand detektiert wird, worauf die Kinasefunktion aktiviert wird. Erste Hinweise für die Kinasefunktion stammen aus der Hefe, wo PASKIN Proteine aus dem Glycogenmetabolismus und der Proteintranslation phosphorylieren kann. In Säugern wurden bislang zwei durch PASKIN phosphorylierte Proteine aus dem Metabolismus entdeckt. Das Erste, wie in der Hefe, ist die Glykogensynthase, und das zweite ist das pankreatische und duodenale Homeobox Protein 1 (PDX-1), ein wichtiger Transkriptionsfaktor für die Insulinexpression. Entsprechend wurde publiziert, dass PASKIN in β-Zellen des Pankreas durch Glukose induziert werden kann. Dies führt zu einer erhöhten Insulinexpression und sekundär zu gesteigerter Insulinsekretion. Neueste Erkentnisse haben auch eine inhibitorische Funktion von PASKIN in der Glukagonsekretion aufgezeigt. Aufgrund all dessen wird angenommen, dass PASKIN nicht nur den Energiehaushalt der Hefe reguliert, sondern auch den von höheren Lebewesen. Des Weiteren wurde auch die Homöostase auf der Ebene des Gesamtorganismus untersucht, wobei unsere Paskin$^{-/-}$ Mäuse verwendet wurden. Nach Fütterung einer Hochfettdiät waren Paskin$^{-/-}$ Männchen vor nachteiligen Erscheinungen des metabolischen Syndroms teilweise geschützt. So zeigten sie ein reduziertes Körpergewicht und bessere Glukose- und Insulintoleranz auf als Wildtyptiere und hatten einen hypermetabolischen Phänotyp in Messungen der indirekten Kalorimetrie. In dieser Arbeit hatten wir uns zum Ziel gesetzt, diesen Phänotypen zu reproduzieren. Tatsächlich führte die Fütterung einer Hochfettdiät (45% kalorisches Fett) zu reduziertem Körpergewicht und besserer Glukosetoleranz. Mit der Fütterung einer Hochfettdiät mit 60% kalorischem Fett konnten wir diese Effekte jedoch nicht verstärken. Auch die indirekte Kalorimetrie und Messungen der Körpertemperatur bestätigten den hyperetabolischen Phänotyp nicht. Wir glauben deshalb, dass PASKIN eine sehr subtile Rolle in der Regulierung des Energiehaushaltes spielt, welche von kleinen Unterschieden in der

Tierhaltung, der Nahrung oder dem Alter beeinflusst wird. Aufgrund dessen haben wir uns vermehrt auf die Beantwortung von molekularen und zellulären Fragen konzentriert: 1) Was ist die Substratspezifität für die PASKIN-Phosphorylierung und was könnten neue Zielproteine im Metabolismus und der Proteintranslation sein? 2) Was könnte der aktivierende Ligand für die PAS-Domäne von PASKIN sein? In dieser Arbeit zeigen wir auf, dass der eukaryotische Elongationsfaktor 1A1 nicht nur ein Interaktionspartner von PASKIN ist, sondern auch phosphoryliert werden kann und damit die Translation *in vitro* verbessert. Um weitere von PASKIN phosphorylierbare Proteine zu entdecken, haben wir ausserdem einen Peptidchip verwendet. Wir bestimmten die Substratspezifität, welche derer der Proteinkinasen A und C sehr ähnlich ist, und entdeckten neue metabolische Zielproteine. Wir konnten ausserdem zeigen, dass PASKIN das ribosomale Protein S6 an den Serinen 235/236 phosphoryliert. Normalerweise wird S6 von p70 S6-Kinasen phosphoryliert. Deshalb gilt diese Phosphorylierung als Indikator für die Aktivität des mTOR-Signaltransduktionswegs und zeigt sich vorwiegend in proliferierenden Zellen. Beim Vergleich von Fibroblasten aus Mäuseembryonen von Paskin$^{-/-}$ und Paskin$^{+/+}$ Mäusen konnten wir jedoch keine Unterschiede in der Proteintranslation, Zellgrösse oder dem Zellwachstum feststellen. Die Proteinkinase C weist nicht nur dieselbe Substratspezifität wie PASKIN auf, sondern phosphoryliert den eukaryotischen Elongationsfaktor 1A1 an genau demselben Threonin wie PASKIN. Deshalb stellten wir uns die Frage, ob PASKIN analog zu der Proteinkinase C, auch Phospholipide als Koaktivatoren benötigt. Tatsächlich fanden wir heraus, dass PASKIN an Phosphatidylinositolmonophosphate bindet, allerdings nicht wie erwartet über die PAS-Domäne, sondern via Kinasedomäne. Daraufhin untersuchten wir den Einfluss von Phosphatidylinositolphosphaten auf die Autophosphorylierung und die Phosphorylierung von Zielproteinen von PASKIN. Interessanterweise induzierten monophosphorylierte Phosphatidylinositole die Autophosphorylierung von PASKIN, wohingegen di- und triphosphorylierte Phosphatidylinositole die Autophosphorylierung inhibierten. Hingegen inhibierten alle getesteten Phosphatidylinositolphosphate die Phosphorylierung von Zielproteinen. Da wir ausserdem entdeckten, dass PASKIN entweder an der Zellmembran oder entlang des Aktinskeletts lokalisiert ist, nehmen wir an, dass PASKIN am Vesikeltransport oder dem Umbau des Zytoskeletts beteiligt sein könnte, und dabei durch die Bindung an Phospholipide reguliert wird.

Abschliessend konnten wir aufzeigen, dass PASKIN durch die Phosphorylierung des Elongationsfaktors 1A1 und des ribosomalen Proteins S6 die Proteintranslation in höheren Lebewesen regulieren kann. Die Bindung an Phosphatidylinositolphosphate eröffnet ausserdem neue Perspektiven und könnte Erklärungen für den metabolischen Phänotyp liefern, so zum Beispiel über die Regulation der Glukagonsekretion oder der Exozytose von

GLUT4 im Muskel. Weitere Befunde über die Expression oder Aktivierung von PASKIN würden mit Sicherheit das generelle Verständnis verbessern, wie PASKIN mechanistisch als Sensor des Energiehaushaltes agieren könnte.

3 SUMMARY

PER-ARNT-SIM (PAS) domains are occurring throughout all kingdoms of life. Bacterial and archaeal PAS domains mainly act as sensory modules of a variety of environmental stimuli either directly by binding a ligand to be sensed or indirectly involving co-factors. Sensing induces conformational changes of the PAS domain that are transduced to effector domains, which are often kinases. In other cases, especially in eukaryotic basic helix-loop-helix transcription factors, PAS domains can act as dimerisation interfaces. PASKIN is conserved from yeast to man and displays the unique combination for eukaryotic proteins of a Per-Arnt-Sim (PAS) domain and a Ser/Thr kinase function. For the PAS domain of PASKIN, a model proposed sensing and binding of a yet unknown metabolic ligand that might activate the kinase domain. For the kinase function, a number of proteins involved in glycogen metabolism and protein synthesis were identified as phosphorylation targets in yeast. For mammalian PASKIN, two targets involved in metabolism have been identified. The first target, similar to yeast, is mammalian glycogen synthase, and the second target is pancreatic and duodenal homeobox protein 1 (PDX-1), an important transcription factor for insulin expression. Accordingly, it has been reported that PASKIN levels are induced in pancreatic β-cells upon glucose stimulation, itself induces insulin expression and secondarily affects insulin secretion. Recent findings also reported an inhibitory function of PASKIN in glucagon secretion, suggesting that PASKIN might regulate energy homeostasis not only in yeast, but in mammals as well. Furthermore, whole body homeostasis has been investigated making use of our *Paskin*$^{-/-}$ mouse model. It has been shown that male *Paskin*$^{-/-}$ mice when fed a high fat diet (HFD) were protected from detrimental effects of the metabolic syndrome. *Paskin*$^{-/-}$ male mice displayed a lower body weight and a better glucose and insulin tolerance than wildtype littermates and showed a hypermetabolic phenotype in indirect calorimetry experiments. Using a similar approach, we herein aimed to reproduce the hypermetabolic phenotype on HFD feeding. Whereas we could see a lower gain of body weight for *Paskin*$^{-/-}$ animals and a better glucose tolerance after 45% fat by calories HFD feeding, we could not increase the effects upon feeding a 60% HFD and failed to see any difference in indirect calorimetry or body temperature. We therefore speculate that PASKIN might play a more subtle role in energy homeostasis that might be affected by housing, diet or age of the animals. We therefore focused on the molecular and cellular level aiming to answer the following questions: 1) What is the substrate specificity and what might be potential targets of metabolism and protein translation for the PASKIN kinase function? 2) What could be the potentially activating ligand for the PAS domain of PASKIN? Herein, we show that the human eukaryotic translation elongation factor 1A1 is a novel interaction partner and kinase

target of PASKIN and is able to increase total translation *in vitro*. To screen for other putative PASKIN phosphorylation targets, we assessed the kinase function on a target peptide microarray. We found a substrate specificity similar to the consensus sequences of protein kinase A (PKA) and C (PKC) and identified new metabolic targets. We further identified phosphorylation of ribosomal protein S6 at serines 235/236. Phosphorylation of S6 usually originates from p70 S6 kinases and is a marker for mTOR activity, and therefore often occurring in proliferating cells. However, comparing *Paskin*$^{-/-}$ versus *Paskin*$^{+/+}$ MEFs we could not observe any differences in total translation, cell size or cell growth. Since PKC is known to phosphorylate eEF1A1 at the same site as PASKIN and shows a similar substrate preference, we wondered wether PASKIN also requires similar phospholipid co-activators as PKC. Indeed, we found that PASKIN binds phosphatidylinositol monophosphates, but not as we assumed to the PAS domain but to the kinase domain. We also investigated the effects of phosphatidylinositol phosphates both on auto- as well as on target phosphorylation *in vitro*. Interestingly, while auto-phosphorylation is induced by the presence of phosphatidylinositol monophosphates and reduced by di- and tri-phosphorylated phosphatidylinositides, target phosphorylation is reduced by the presence of all phosphatidylinositides tested. Since we found that PASKIN mainly localises along the cytoskeleton, either at the cell membrane or along stess fibers, this suggests that PASKIN might be involved in vesicular transport or cytoskeletal dynamics and its activity might be regulated accordingly dependent on phospholipid binding.

Conclusively, the identification of eEF1A1 and S6 as novel mammalian phosphorylation targets confirmed a role for PASKIN in the regulation of protein translation. The observation that PASKIN can bind phosphatidylinositides opens novel perspectives for future research as well, and might be connected to a metabolic phenotype by interfering with glucagon granule secretion or GLUT4 exocytosis. Further experiments to find conditions upregulating PASKIN and the identification of an activating ligand would certainly help to understand the general mechanistics of PASKIN as a sensor of energy homeostasis.

4 INTRODUCTION

4.1 Per-ARNT-Sim (PAS) domains

As cells have to adapt to a dynamic environment, nature evolved sophisticated systems to sense environmental changes. To adapt to new conditions, sensors activate intracellular signaling cascades and allow a cell to adjust to the altered environment. A major class of sensors is represented by Per-ARNT-Sim (PAS) domains. They are named after three domains of homologous primary sequence (1). These basic helix-loop-helix (bHLH) proteins are the *Drosophila* protein period (per), a component of the circadian clock (2), the aryl hydrocarbon receptor nuclear transporter (ARNT), a transcription factor dimerising with the aryl hydrocarbon receptor (AHR) (3) or hypoxia-inducible factor α-subunits (HIFα) (4), and single-minded (sim), a protein involved in neurogenesis related to Down syndrome (5). Since the discovery of a common domain structure, many additional PAS proteins added up to the list, deriving from all kingdoms of life. Currently, the Pfam database includes more than 33.000 PAS domain annotations (version 24.0, October 2010) (6).

Structurally, the PAS fold comprises a central five stranded β-sheet flanked by several α-helices, forming a structure resembling a baseball glove with, in some cases, a ligand or cofactor bound to the center (Fig.1). They often occur as tandem PAS repeats of 70-130 amino acids in a stretch of ~275 residues and are termed PAS A and PAS B (7). Functionally, PAS domains either serve as dimerisation interfaces or act as sensors for various stimuli. In eukaryotic systems, bHLH-PAS transcription factors are the most studied PAS domain containing proteins. For example the HIF-system needs protein-protein interaction of a HIFα and ARNT subunit to form a functional bHLH-transcription factor that induces target gene expression and allows the cell to adapt to the hypoxic environment (8).

PAS-domains, however, also serve as sensory modules. Sensory PAS-domains induce conformational changes that alter the activity of transmitter domains. These changes are induced by the agent to be sensed and activate other domains of the protein such as histidine kinases, serine/threonine kinases, ion transporters or transcription factors (9). For the aryl hydrocarbon receptor, also known as dioxin receptor, the ligand to be sensed can bind directly to the PAS domain (3), but many cases involve co-factors. NPAS2 is a bHLH transcription factor that requires a heme co-factor to sense its ligand, carbon monoxide (10). CO acts as a neurotransmitter in the forebrain and is inhibitory to NPAS2 transcription factor function (11). A fascinating example for another co-factor containing PAS domain linked to a kinase function is represented by plant phototrophins (phot). Blue light for plant phototropism is sensed by a flavin mononucleotide (FMN) co-factor in the PAS domains (12). Blue light

induces covalent linkage of FMN to a cysteine residue, and conformational changes activate the serine/threonine kinase domain (13). Other sensors include the mammalian voltage gated potassium channel HERG with a PAS domain that is able to sense membrane potentials (14), or the flavoprotein Aer of *Eschrichia coli* that senses changes in redox potential (15).
Whereas in prokaryotes and archaea many PAS domains are linked to kinase domains, mostly histidine kinases, PAS kinase or PASKIN is the only mammalian representative linking a PAS fold to a serine/threonine kinase domain.

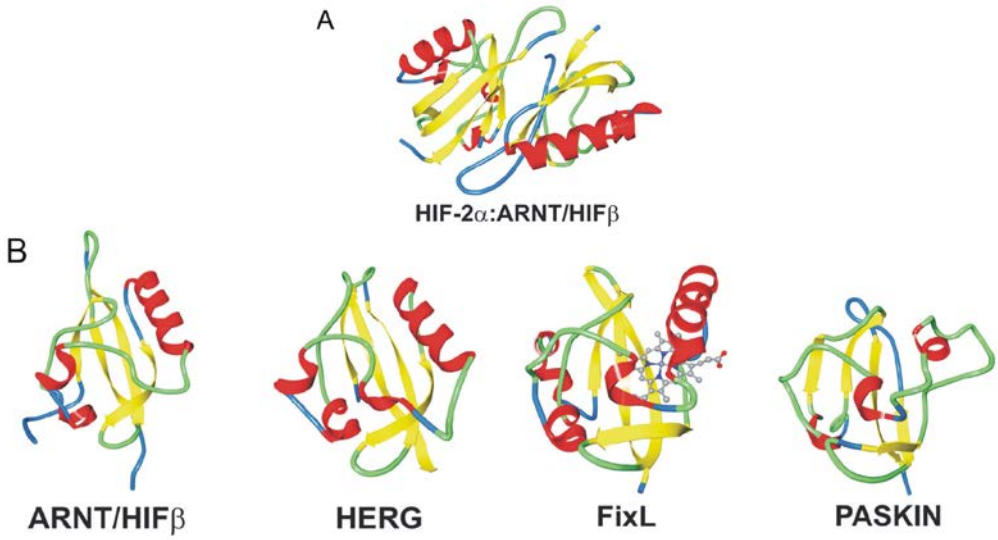


Fig. 1. Structures of various PAS domains. (A) PAS domain heterodimerization between HIF-2α and ARNT/HIFβ. (B) Structural similarity between various PAS domains. Structures are based on the following Protein Data Bank coordinates: HADDOCK structure of HIF-2α:ARNT/HIFβ heterodimer (2A24) (16);NMR structure of ARNT/HIFβ (1X0O) (16) and PASKIN (1LL8) (17); Structure of HERG (1BYW) (14) and FixL including the heme group (1D06) (18).

4.2 Human PASKIN, a PAS domain containing Ser/Thr kinase

FixL is an oxygen sensor of nitrogen fixating *Bradyrhizobium* and *Rhizobium* species, the bacteria living in symbiosis with *Fabaceae* in root nodules. In the FixL PAS domain, oxygen can bind to ferrous iron within a heme moiety in the center of the PAS domain. Binding inactivates the histidine kinase function of FixL and thereby interrupts downstream signaling that would lead to transcription of genes required for nitrogen fixation (19). Performing a blast search of the PAS domain, our group found an uncharacterised protein showing relatively high similarity to the PAS domain of FixL (20). Similarity searches of the uncharacterised mammalian protein suggested a serine/threonine kinase domain linked C-terminally to the PAS domain. This protein with a PAS and a kinase domain was therefore called PASKIN. At the same time, the group of Steven McKnight identified PASKIN by similar blasting procedures, naming it PASK (21). Due to the fact that the name PASK is

already used for proline- and alanine-rich Ste20 related kinase (22), the name PASKIN will be used throughout this text.

4.2.1 PASKIN gene structure

PASKIN is encoded by a single copy gene that is conserved throughout mammalian species. The human *PASKIN* gene is located on chromosome 2 and consists of 18 exons coding for a protein of 144 kDa (20). Interestingly, only 1 kb upstream of the first exon of *PASKIN*, the gene for protein phosphatase 1, regulatory subunit 7 (*PPP1R7*) encodes a protein named Sds22 (20).

A similar intron/exon structure as for the human *PASKIN* gene was found for the mouse gene (20). To investigate PASKIN expression, a *Paskin* knockout mouse was generated by replacing exon 10-14 with a *β-geo* cassette, expressing β-galactosidase as a reporter (23). *Paskin*$^{-/-}$ mice showed no difference in viability, fertility, growth or behaviour. Analysis of Paskin expression in different organs in human (20) and mouse (23) tissues by Northern blotting revealed a high expression of Paskin in the testis and lower expression in brain and thymus, whereas general expression of the gene seemed to ubiquitous at very low levels. The Northern blot data were confirmed later on by quantitative real time PCR, adding bone marrow to the list of Paskin expressing tissues (24).

4.2.2 The PAS domain of PASKIN

At its N-terminus, PASKIN contains two PAS domains named PAS A and PAS B. The PAS A domain of PASKIN exhibits a typical PAS fold with a five-stranded β-sheet flanked by several α-helices (Fig. 1), as demonstrated by NMR methods (17). However, despite the structural relation, we found heme bound to FixL but not to PASKIN (unpublished data), and there is ample evidence to rule out a role for PASKIN in direct oxygen-sensing in mammals. Nevertheless, the Gardner Lab identified synthetic ligands that bind to PAS A of PASKIN, implying that there might exist yet unidentified endogenous ligands (17).

Functionally, the PAS-domain of PASKIN seems to inhibit the kinase function as the catalytic activity of a N-terminal deletion of the PAS domain increased PASKIN activity by a factor of five (21,17). Additionally, by adding increasing amounts of purified PAS A domain, kinase activity was decreased in *trans* (17). Derived from the structural analysis of the PAS domain, it could be shown that residues in the flexible FG-loop were responsible for kinase binding (17). Altogether, these results combined to a model where in unbound state the PAS domain was inhibitory to the kinase function, whereas binding of a ligand would induce

conformational changes in the PAS fold leading to de-repression of the kinase domain and allow transmission of the sensory signal (Fig. 2).

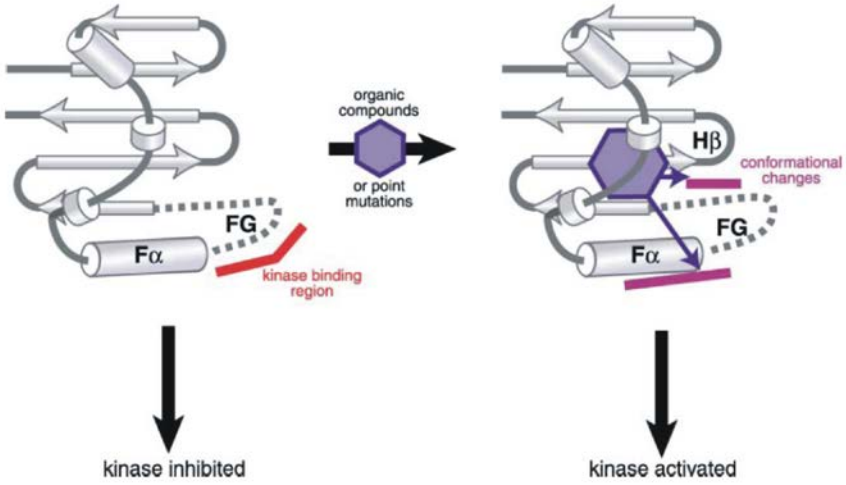


Fig. 2. Activation of PASKIN by binding of a small organic ligand. The flexible FG-loop of the PAS domain of PASKIN binds the kinase domain, inhibiting the kinase *in cis*. Upon ligand binding, conformational changes are induced and binding is lost leading to de-repression the kinase domain (17).

4.2.3 The kinase domain of PASKIN

PASKIN belongs to the family of serine/threonine kinases and is a member of the CamK superfamily (25). Very recently, the crystal structure of the kinase domain of human PASKIN has been solved (PDB: 3dls) (26). It shows a typical two-lobe fold with an N-terminal five stranded β-sheet and a C-terminal α-helical domain (Fig.3). Many kinases are regulated through phosphorylation of an activation loop (27). This loop is thought to stabilise an active conformation by forming hydrogen bonds with both the N-terminal β-sheet and the C-terminal α-helical domains when phosphorylated. PASKIN indeed shows such an activation loop structure (26). An earlier report identified two autophosphorylation sites at T1161 and T1165 (21), leading to the hypothesis that these residues might be critical for activation loop functionality. However, functional assays along with the three dimensional structure do not attribute functionality to this autophosphorlyation, as T1161A mutant PASKIN phosphorylates targets *in vitro* to the same extent as wildtype PASKIN (26). Another function of the activation loop is its involvement in substrate binding and catalysis. In the activation loop of PASKIN, a conserved catalytic arginine (R1127) precedes a conserved aspartate (D1128) residue. This feature classifies PASKIN as a RD kinase. Typically, a (auto-) phosphorylated moiety of the activation loop directly interacts with this conserved RD motif

to stabilise the active conformation (27). In PASKIN, however, the structure and noncovalent binding partners of the RD-residues support a phosphorylation-independent stabilisation (26).

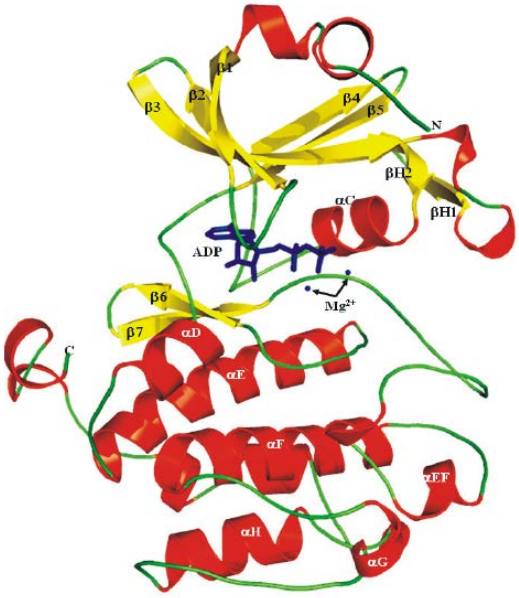


Fig. 3. Kinase domain of PASKIN. The X-ray structure of the kinase domain of PASKIN co-crystallised with ADP (PDB: 3dls) shows a typical fold with a β-sheet and a C-terminal α-helical domain and comprises two Mg^{2+} ions. The RD-motif is located in the loop between β6 and β7. The auto-phosphorylation sites T1161 and T1165 are located on the αE helix. (26)

The structure of PASKIN confirmed the previously suggested presence of an ATP binding site comprising T1032. This residue is also involved in binding of one of two Mg^{2+} ions present in the kinase domain (26). To determine the substrate specificity, a combinatorial peptide library screen was performed that suggested a substrate specificity relative to the phosphorylatable serine or threonine as follows: -5 position: histidine or arginine, -3 position arginine and -2 histidine, arginine or lysine (26). For *in vivo* situations, however, not many direct PASKIN kinase targets have been published. First insight came from yeast Psk proteins which target proteins of the glycogenic and glycogenolytic pathways as well as proteins responsible for protein synthesis (28). Human phosphorylation targets published to date include glycogen synthase (29), pancreatic and duodenal homeobox protein 1 (30) and eukaryotic elongation factor 1A1 (31).

4.3 PASKIN in yeast

Saccharomyces cerevisiae possesses two orthologous PASKIN genes, *Psk1* and *Psk2*. Primary sequence analysis shows 71% homology among the two isoforms, with 90% homology in the kinase domain and 81% in the PAS domain (32). First insights into Psk function were gained from yeast with a deletion in both *Psk1* and *Psk2*. While under standard growth conditions no phenotype was observed, *Psk* deletions showed unability to grow on galactose as only energy source at high temperature (39°C) (28). A genomic DNA library screen was performed, aiming to find targets rescuing this growth defect under stress conditions. Besides *Psk1* and *Psk2*, most suppressors of the phenotype affected protein synthesis, either by increasing protein synthesis or indirectly by modifying RNA transcription. The same publication also addressed a biochemical approach that revealed novel Psk kinase targets (28). Three of the targets found, namely Caf20, Tif11 and Sro9 are involved in protein translation and will be discussed in more detail in section 3.5.1. Another target protein, Ugp1, is involved in glucose metabolism. UDP-glucose pyrophosphorylase (Ugp1) produces UDP-glucose, an activated substrate for glycogen and cell wall glucan biosynthesis. Interestingly, a yeast strain expressing an unphosphorylatable form of Ugp1 (S11A) displays a similar phenotype to the $Psk1^{-/-}Psk2^{-/-}$ double mutant on galactose energy source at high growth temperature (28,33). These findings led to the proposal that Psk proteins phosphorylate Ugp1 in order to upregulate cell wall biosynthesis in favor of glycogen synthesis. This regulation seems to depend on the subcellular localisation of Ugp1, where phosphorylation at S11 does not alter the activity of Ugp1 but accumulates the protein at the outer cell membrane (33). In line with the Ugp1 related function of Psk in *Saccharomyces cerevisiae*, Psk1 is also invovled in the regulation of the cell wall damage response in *Candida albicans* (34). Psk1 induces the expression of genes directly involved in cell wall repair, and hence, a $Psk1^{-/-}$ strain showed hypersensitivity to cell wall damage induced by the antifungal drug caspofungin.

4.4 Energy homeostasis

Dietary food intake supplies living organisms with energy which is either used for consumption, growth or storage. The system maintaing the availability of energy is highly sophisticated and tightly controlled. Energy homeostasis ensures the constant supply of organs, mainly muscle and brain, with rapidly available energy and regulates body temperature and storage of excess energy. The main storage forms of energy are glycogen in muscle and liver and triglycerides in adipose tissue and liver.

The master regulator of glucose homeostasis is the peptide hormone insulin. After a diet, glucose levels in the blood increase and stimulate the β-cells of the islets of Langerhans to secrete insulin into the blood stream. Insulin stimulated uptake of glucose from the blood occurs predominantly into liver, muscle and adipose tissue. In these tissues, glucose uptake leads to increased glycogen and fatty acid synthesis and inhibition of degradative processes and gluconeogenesis. The counter player of insulin is glucagon, produced in the α-cells of the pancreatic islets. Glucagon is secreted upon a drop of blood glucose levels. The degradative effect of glucagon can be observed in the liver, where it leads to the breakdown of glycogen and release of glucose into the blood stream. Additionally, glucagon stimulates gluconeogenesis in the liver and lipolysis in adipose tissue.

An imbalance of the tightly regulated system is a major health concern especially for Western countries. Western diets consist of high caloric meals combined with reduced physical activity. This imbalance results in what is called metabolic syndrome, characterised by obesity and eventually development of type 2 diabetes and cardiovascular disease. Estimates for 2010 by the International diabetes federation show that as much as 11.3% of the Swiss and 8.6% of the European population of 20-79 years of age suffer from diabetes mellitus, causing 3350 deaths per year in Switzerland and 634.000 deaths per year in Europe (35).

Diabetes mellitus is classified into two types, type 1 and type 2 diabetes. Type 2 diabetes is characterised by insulin resistance in peripheral tissues. Reduced insulin stimulated blood glucose uptake into tissues leads to hyperglycemia, and as a reaction to elevated blood glucose levels, pancreatic β-cells further secrete insulin. On long term, this leads to β-cell exhaustion, where patients enter a vicious circle of both peripheral insulin resistance and reduced insulin secretion. In contrast, type 1 diabetes is characterised by a β-cell malfunction. Type 1 diabetic patients are not able to produce and secrete sufficient insulin, resulting in hyperglycemia, whereas insulin stimulated (by insulin injection) peripheral glucose uptake is normal.

4.4.1 PASKIN and glucose homeostasis

PDX-1 is the major transcription factor for preproinsulin expression in pancreatic β-cells. Upon glucose stimulation, PDX-1 gets phosphorylated in a phosphatidylinositol 3-kinase dependent manner, leading to import of PDX-1 into the nucleus and subsequent induction of insulin gene expression (36). First investigations on Min6 cells and isolated rat islets demonstrated that also PASKIN is involved in the expression of preproinsulin (37). PASKIN mRNA and protein were induced upon glucose stimulation in pancreatic β-cells. When overexpressed, PASKIN mediated the onset of the preproinsulin promoter, usually occuring under elevated glucose levels, already under low levels of glucose in culture media. Indeed, a direct connection between PDX-1 and PASKIN was evidenced. PDX-1 promoter activity was higher when PASKIN was overexpressed and lower when PASKIN was knocked down (37). A follow up report demonstrated that PDX-1 is also an *in vitro* phosphorylation target of PASKIN. Hence, it was speculated that the transcription of the insulin gene might be due to increased translocation into the nucleus upon PASKIN mediated phosphorylation (30).

In contrast to these results, our lab could not confirm increased PASKIN expression in islets of Langerhans in mice stimulated with high glucose (24). Even though the expression of insulin was slightly lower in *Paskin*$^{-/-}$ islets compared to *Paskin*$^{+/+}$ islets, the differences were neglectable and no difference in inducibility upon glucose stimulation could be observed. In contrast, secreted insulin from the supernatant of cultivated islets of Langerhans were even higher in *Paskin*$^{-/-}$ cells (24).

Diabetic patients develop hyperglycemia and hyperlipidemia due to insulin resistance in peripheral tissues. Elevated serum glucose and fatty acid levels trigger pancreatic β-cells to produce and secrete more insulin, which leads in long term to β–cell exhaustion and apoptosis, an effect which is called glucolipotoxicity (38-39). It was found that palmitate could inhibit insulin expression (40). As PASKIN levels were increased in glucose stimulated pancreatic β-cells, expression levels of islets stimulated by glucose and palmitate simultaneously were analysed. Interestingly, such mimicking of glucolipotoxicity reversed the effect of increased PASKIN expression to levels comparable to unstimulated cells (41). If, as previously suggested, PASKIN is essentailly involved in insulin gene expression via

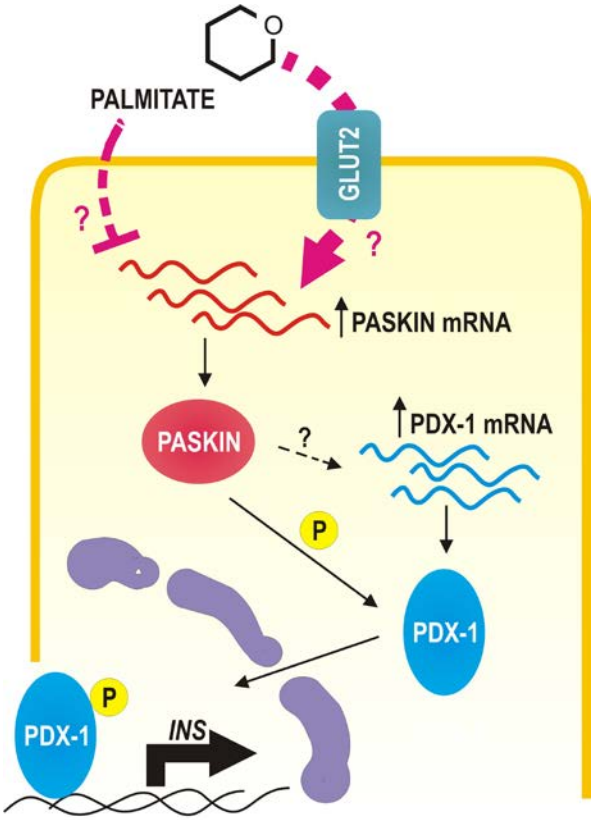


Fig. 4. Model for PASKIN function in insulin expression of pancreatic β-cells. Glucose stimulated pancreatic β-cells induce PASKIN mRNA expression. Upregulation of PASKIN leads to increased expression of the transcription factor PDX-1. Furthermore, PASKIN phosphorylates and thereby eventually triggers the nuclear import of PDX-1, where the transcription factor activates insulin gene expression. In glucolipotoxic situations, palmitate inhibits PASKIN mRNA expression and inhibits the pathway leading to expression of insulin despite stimulatory glucose concentrations.

PDX-1 (37), these data suggest that the reduced PASKIN levels are responsible for palmitate inhibition of insulin gene expression. This hypothesis was further supported by the fact that overexpression of PASKIN circumvented the inhibition of insulin gene expression upon palmitate stimulation.

Hypothesizing that PASKIN acts as a metabolic sensor, in pancreatic α-cells PASKIN effects opposite to the β-cells are expected. Indeed, isolated islets of Langerhans of *Paskin*$^{+/+}$ mice showed decreased glucagon secretion at elevated glucose levels, whereas islets of *Paskin*$^{-/-}$ mice secreted glucagon almost to the same extent as in a low glucose situation. Supportingly, PASKIN overexpression in cultivated human islets inhibited glucagon secretion (G. da Silva Xavier, personal communication).

Despite our findings of low PASKIN expression in islets of Langerhans and a lack of stimulatory PASKIN expression (24), altogether these data suggest a role in whole body glucose homeostasis by regulating insulin and glucagon levels. To complement a role for PASKIN in whole body energy homeostasis, several findings imply a PASKIN function in

peripheral tissues. Early findings showed – analogous to the findings in yeast – that human PASKIN interacts with and phosphorylates rabbit glycogen synthase *in vitro* and that phosphorylation of glycogen synthase could be inhibited by the presence of glycogen (29). Going into more depth, the laboratory of Jared Rutter performed *in vivo* experiments based on high fat diet induced obesity (42), making use of our *Paskin*$^{-/-}$ mouse model (23). Whereas glucose and insulin tolerance tests were equal for *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ animals on normal chow, *Paskin*$^{-/-}$ mice on high fat diet showed better glucose tolerance than wildtype animals. This finding went in line with observations of the body weight, since *Paskin*$^{-/-}$ animals were partially protected from high fat diet induced obesity, and was was reconfirmed by the observation of a fatty liver phenotype of *Paskin*$^{+/+}$ animals. Together with the incidence that *Paskin*$^{-/-}$ mice also showed increased O_2 consumption and CO_2 production in indirect calorimetry, this led to the assumption that *Paskin*$^{-/-}$ mice were hypermetabolic. Several experiments aimed to support this phenotype on the cellular level. Permeabilised *Paskin*$^{-/-}$ soleus muscles showed higher ATP synthesis rate and a knockdown of PASKIN in L6 myoblast cells showed increased glucose and palmitate oxidation along with slightly elevated ATP-levels. Yet, these data lack mechanistical proof, as there was no difference in mitochondrial number nor in AMPK or S6 kinase activity nor in PGC1α levels. The hypermetabolic phenotype is also challenged by findings of our lab, as we could not show differences in body weight gain on high fat diet and no hypermetabolic phenotype was observed in indirect calorimetry experiments.

4.5 Protein synthesis

Cell growth requires large amounts of nutrients and is energy consuming. As proteins are a major component of total cellular mass, protein synthesis is increased in growing cells. This process of protein translation is complex and tightly regulated.

For an efficient increase of overall protein synthesis, several criteria need to be fulfilled, namely the stimulation of cells by growth factors, the supply with nutrients (especially amino acids) and the energy status. These growth stimuli are mainly integrated through the serine/threonine kinase ‘mammalian target of rapamycin’ (mTOR). Two complexes of mTOR exist, complex1 (mTORC1 or mTOR-raptor complex) and complex 2 (mTORC2 or mTOR-rictor complex) (43). The function of mTORC1 has been extensively studied, as this complex can be directly inhibited by the drug rapamycin (43). Complex 1 is mainly involved in processes increasing overall protein translation whereas complex 2, that cannot be inhibited by rapamycin, seems to play a role in actin cytoskeleton dynamics (44).

Among nutrients activating mTORC1, amino acids play a central role (45). Amino acid starvation of cultured cells, especially leucine starvation, results in rapid dephosphorylation of the mTORC1 effectors S6K1 and 4E-BP1 (46). Readdition of amino acids leads to reactivation of this pathway, thereby linking amino acid availability to overall protein synthesis (46). For cellular growth the energy status is at least as important as nutrient availability. The main cellular sensor of the energy status is AMP-activated protein kinase (AMPK). As the name of the protein implies, high levels of AMP activate the kinase, thus sensing the AMP/ATP ratio. Consequently, activated AMPK stimulates ATP-generating processes and inhibits energy consuming processes. With respect to mTOR signalling, the protein synthesis with its high energy costs is reduced when AMPK is activated. This happens through activating phosphorylation of tuberous sclerosis protein 2 (TSC2) an inhibitor of the mTOR pathway (47).

Nutrients and energy are not the only prerequisites for cell growth, but cells also need growth factor stimuli. Activation of the mTOR pathway after growth factor stimuli (see Fig. 5) happens via the PI 3-kinase (PI3K) pathway (48). Growth factor receptors such as the insulin receptor recruit PI3K via insulin receptor substrate (IRS) to the plasma membrane, where PI3K converts PtdIns(4,5)P_2 (PIP2) to PtdIns(3,4,5)P_3 (PIP3) (49). Increased levels of PtdIns(3,4,5)P_3 recruit PDK1 and Akt to the membrane, leading to activation of Akt (50). Akt in turn, signals by inhibtory phosphorylation to tuberous sclerosis proteins (TSC1-TSC2) (51) activating mTOR. Activated mTORC1 regulates protein translation by activating p70 S6 kinase (S6K) and phosphorylating 4E binding protein (4E-BP). Additionally, mTOR regulates transcription factor recruitment to the nucleus transcribing genes for ribosome biogenesis or genes involved in metabolic pathways

Regarding a potential function of PASKIN in intracellular signaling related to protein translation, it is interesting to note that increased AMPK activity and reduced mTOR pathway activity show a phenotype with resistance to diet-induced obesity as demonstrated by transgenic and pharmacologic strategies (52-53). Rutter and colleagues performed Western blots of *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ samples from liver and gastrocnemius muscle using antibodies against phospho-AMPK and phospho-S6K. However, no difference in the activation of these two pathways could be observed (42). Still, there is some evidence that PASKIN might be involved in translation especially by phosphorylated targets found in yeast and mammals, even though a pathway activating PASKIN remains unidentified.

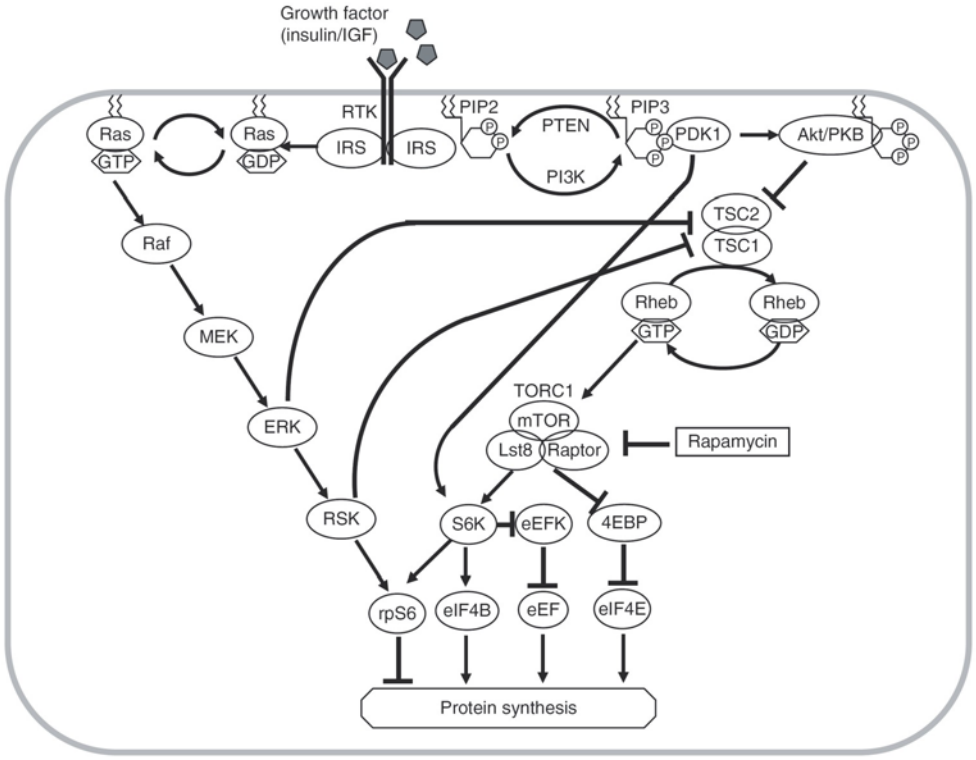


Fig. 5. Growth factor signaling in translation. A central role is attributed to the PI 3-kinase and Akt dependent activation of the mTOR pathway, signaling to S6K and ribosomal protein S6. See text for details. (54)

4.5.1 PASKIN function in protein translation

Translation consists of three major steps, namely initiation, elongation and termination. The most regulated step is translation initiation, which involves the ribosomal 40S and 60S subunits as well as several eukaryotic translation initiation factors (55). For the assembly of a translationally active ribosome, complexes of the 40S ribosomal subunits and initiation factors form. This involves the binding of eIF4E to the 5'-cap structure of the mRNA, which is a tightly controlled and rate limiting step. The control mechanism is mediated by the 4E binding protein (4E-BP). Phosphorylation of 4E-BP by mTOR releases eIF4E and allows ribosome assembly at the cap structure (56). Once this complex is formed, the mRNA is scanned until a start codon is recognised. With the addition of a 60S subunit to form the full 80S ribosomal complex, translation initiation is terminated and elongation starts.

Rutter and colleagues identified novel Psk phosphorylation targets in yeast (28) that are mainly involved in mRNA binding and translation initiation: Caf20p, eIF1A and Sro9p. Caf20p (Cap-Associated Factor 20 protein/p20) is a small cap-associated protein that can be phosphorylated at several positions (57). Since Caf20p was shown to interact with eIF4E in a competitive way against eIF4G binding and therefore negatively regulates cap-dependent translation initiation, it may functionally be equivalent to 4E-BP of mammalian cells (58). In

the screening for phosphorylation targets of Psk2, Caf20p was only phosphorylatable *in vitro* in presence of its binding partner eIF4E at two distinct phosphorylatable serine residues (28).
The eukaryotic initiation factor 1A. (eIF1A) mediates the transfer of Met-tRNA to the 40S ribosomal subunit (59). *In vitro* analysis revealed phosphorylation at S125 (28), however, no phosphorylation-dependent function of eIF1A has been reported so far.
The third target of Psk2, Sro9p, has been shown to bind to RNA and associates with translating ribosomes (60). A more recent report involved Sro9p in several complexes involved in transcription and translation (61). Therefore, it is hypothesized that Sro9p is recruited to nascent mRNA already during transcription, keeps the interaction during mRNA export up to the translation in ribosomes. Even though phosphorylation has been shown previously in high throughput screenings (62), the physiological function awaits further investigations.

4.5.2 Eukaryotic elongation factor 1A1

Whereas first hints on PASKIN function involve mRNA binding and translation initiation, we identified the eukaryotic elongation factor 1A1 (eEF1A1) as novel PASKIN phosphorylation target (31). In the elongation phase, amino-acylated tRNAs (aa-tRNAs) supply translating ribosomes with the respective amino acids. The recruitment of amino-acylated tRNAs to the acceptor site of the ribosome is controlled by eEF1 consisting of the two subunits eEF1A and eEF1B. The G-protein eEF1A binds the acceptor site of the ribosome as a complex of eEF1A-GTP-aa-tRNA. Subsequent hydrolysis of GTP releases eEF1A-GDP from the ribosome for recycling to eEF1A-GTP. Two different isoforms of eEF1A are known, eEF1A1 and eEF1A2. They share 92% sequence identity (63), but have a different tissue distribution. In rat, eEF1A1 is expressed in brain, liver and spleen, whereas eEF1A2 is predominantly found in heart, brain and muscle (64-65).
A function of eEF1A distinct from its role in aa-tRNA recruitment involves the cytoskeleton. It has been shown, that efficient protein synthesis is relying on an intact actin cytoskeleton (66). Indeed, eEF1A interacts with F-actin directly and a GFP-eEF1A fusion protein colocalises with phalloidin (actin stain) at the edges of the cell (67). Hence, the cytoskeleton might concentrate the translational machinery for efficient protein synthesis. Interestingly, we could show a colocalisation of PASKIN with eEF1A in the midpiece of the sperm tail, where cytoskeletal structures are highly abundant (31).
Eukaryotic elongation factor 1A is also a phosphoprotein. Large scale proteomics studies have shown tyrosine phosphorylation at several residues conserved in both eEF1A1 and eEF1A2 (68-70). However, as many as 11 residues differing in primary sequence between

eEF1A1 and eEF1A2 involve serine or threonine residues (63), implying a tissue-specific regulatory function for Ser/Thr kinases. We have shown, that PASKIN phosphorylates eEF1A1 at T432 and increases the elongation activity *in vitro* (31). What other kinases are known to phosphorylate eEF1A1, and what physiological consequences of phosphorylation are known? Most interestingly, protein kinase Cδ (PKCδ), a kinase that is fully active only upon binding of phospholipids, phosphorylates eEF1A1 similar to PASKIN at T432 (71). Upon phosphorylation, elongation activity was increased by threefold and was suggested to be due to increased GDP/GTP exchange activity in the recycling step of eEF1A1 (72-73). Another kinase phosphorylating eEF1A1 is Rho-associated kinase, however, the phosphorylated peptides identified did not include T432 (74). A third kinase, C-Raf both interacts as well as phosphorylates eEF1A1. Cells stimulated with interferon α induced apoptosis, increased the content of eEF1A1 and exhibit phosphorylation of serine and threonine residues, identified by anti-phosphoserine/threonine antibodies. However, the phosphosites were not experimentally determined, but predicted *in silico* by analysing the structure of eEF1A1. Accordingly, potential phosphorylation could occur at S18, S157, T242, S316, S383 and T432, the site phosphorylated by PASKIN (75).

4.5.3 Ribosomal protein S6

Herein, data identifying ribosomal protein S6 (rpS6) as a novel PASKIN phosphorylation target will be presented. Therefore, this section summarises current literature about phosphorylation of rpS6.

In eukaryotic cells, the ribosomes consist of two subunits. The small 40S ribosomal subunit is a complex of a single RNA molecule (18S rRNA) and 33 proteins, whereas the large 60S subunit is composed of three RNAs (5S, 5.8S and 28S rRNA) and 46 proteins. Among the proteins, ribosomal protein S6 (rpS6) has attracted most attention. It is located in the 40S ribosomal subunit and can be phosphorylated. This implied that translational activity could be regulated by kinases via rpS6.

Phosphorylation of rpS6 was first detected in rabbit reticulocytes (76) and is located at five clustered residues at the C-terminus, S235, S236, S240, S244 and S247 (77). p70 ribosomal protein S6 kinases (S6K1, S6K2) phosphorylate all five serine residues (77) and are induced through mTOR complex 1 (78). Other kinases phosphorylating rpS6 are the p90 ribosomal S6 kinases (p90RSK, see Fig. 5). They phosphorylate rpS6 at S235 and S236 and are activated by the extracellular signal-regulated kinase (ERK) pathway (79). Interestingly, ribosomal protein S6 is dephosphorylated by protein phosphatase 1 (80), of which the gene for its regulatory subunit SDS22 is located just 1 kb upstream of the *PASKIN* gene (20).

The classical idea how phosphorylation of rpS6 could influence protein translation is based on translation of 5'-terminal oligopyrimidine (5'-TOP) mRNAs. 5'-TOP mRNAs are a special set of mRNAs defined by two criteria. The structural characteristics of a 5'-TOP sequence following the mRNA cap and the functional effect of growth-associated translational regulation by selective recruitment to polysomes (81). Most of the 5'-TOP mRNAs are coding for components of the translational machinery itself. Thus, all 80 ribosomal proteins (including rpS6) (81) and also the elongation factors (including eEF1A1 (82)) are encoded by TOP genes (83). For rpS6, this translational regulation coincides with phosphorylation of rpS6. Therefore it was proposed and widely accepted that phosphorylation of rpS6 increases the affinity of ribosomes to incorporate 5'-TOP mRNAs (84). However, several findings challenge this hypothesis of direct interdependence of rpS6 phosphorylation and 5'-TOP mRNA expression, as shown in the following paragraphs describing relevant *in vivo* mouse models.

First, a conditional knockout of the *RPS6* gene in mouse liver showed that ribosomal protein S6 was indispensible for proliferation of hepatocytes after a partial hepatectomy (85) and T-lymphocytes displayed a cell cycle arrest due to failure of proliferation (86). Upstream of ribosomal protein S6, mice deficient for p70 S6 kinases displayed growth-related phenotypes, as well. Disruption of *S6K1* revealed mice with growth defects manifested in reduced body weight already at embryonic stage all through to adult mice (87) and *S6K1*$^{-/-}$/*S6K2*$^{-/-}$ mice even exhibited perinatal lethality (88). However, MEFs derived from *S6K1*$^{-/-}$/*S6K2*$^{-/-}$ mice still showed incorporation of 5'-TOP mRNAs into polysomes. This finding challenges the idea of a direct effect of rpS6 phosphorylation to 5'-TOP mRNA translation.

To investigate the physiological function of rpS6 phosphorylation directly, mice unable to phosphorylate rpS6 were generated. In these knockin mice (designated *rpS6*$^{P-/-}$), all five phosphorylatable serines of rpS6 were substituted with alanines (89). Neither in liver nor in cultured MEFs, differences in expression of 5'-TOP mRNAs between *rpS6*$^{P+/+}$ and *rpS6*$^{P-/-}$ material could be observed, and global protein synthesis rate assessed by [^{35}S]-Met and [^{35}S]-Cys incorporation surprisingly even showed a higher rate in *rpS6*$^{P-/-}$ MEFs. *In vivo* experiments showed that cell size might be regulated by rpS6 phosphorylation in certain cell types. In *rpS6*$^{P-/-}$ mice, pancreatic β-cells were smaller and displayed reduced plasma and pancreatic insulin with impaired glucose tolerance. A follow up study focused on the exercise performance (90). Knock-in mice performed worse in muscle strength and endurance exercises than *rpS6*$^{P+/+}$ mice. This could partially be explained by reduced soleus muscle mass due to smaller myofibers, suggesting again a defect of the reduced 'cell size' type. Muscles of *rpS6*$^{P-/-}$ mice also displayed impaired energy storage manifested in reduced levels of ATP and phosphocreatine and increased levels of glycogen. The latter could be explained by increased glycogen synthase activity.

4.6 Other PASKIN functions: testis and regulation of ventilation

In testis, a high expression of PASKIN was repeatedly reported (20,23-24). However, reproduction rate of *Paskin*$^{-/-}$ mice was not affected (23). To further investigate the high PASKIN levels, X-Gal stainings for β-galctosidase reporter gene activity in testis derived from *Paskin*$^{-/-}$ mice were performed and restricted the expression to the seminiferous tubules (23,31). Cross-sections confirmed PASKIN expression in the outermost layer of the tubules corresponding to spermatocytes, spermatids and spermatozoa (23). The upregulation of PASKIN expression in postmeiotic spermatids suggest a role for PASKIN in mature spermatozoa. Indeed, in ejaculated human sperm cells, PASKIN is localised to the midpiece of the sperm tail (31). However, a lack of PASKIN affects neither fertility, sperm production nor motility (23). As sperm maturation is a very dynamic process involving genetic rearrangement, meiosis, differentiation and spermatozoa expend energy on motility, no function could yet be attributed to PASKIN. Noteworthy, the midpiece of the sperm tail is enriched in mitochondria and cytoskeletal structures, that might imply a potential metabolic function in moving spermatozoa.

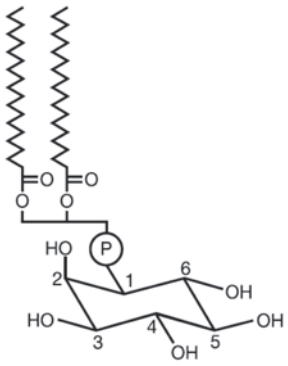
One publication suggested a function for PASKIN in hypoxic ventilatory response (91). *Paskin*$^{-/-}$ females but not males had increased respiratory frequency and tidal volume in an acute hypoxic setting (up to 70 min, decrease to 8% O_2) and decreased tidal volume in a chronic (3 days, 10 % O_2) hypoxic setting. This might partially be explained by elevated levels of catecholamines in the brainstem of female *Paskin*$^{-/-}$ mice.

4.7 Phospholipids

A large amount of the cell is composed of membranes. They build up closed compartments, namely the nucleus, mitochondria (with inner and outer membrane), the endoplasmic reticulum, the Golgi apparatus and the cellular (outer) membrane. Small vesicles such as exosomes, endosomes, lysosomes or other transport vesicles complement the set of static membranes with a dynamic component. All membraneous compartments display specific hallmarks allowing trafficking and signaling at the appropriate intracellular site. One component are phospholipids, the building blocks of membranes. Different phospholipids vary in their occurrence among the inner and the outer layer of a membrane as well as among the different organelles both in composition and relative amounts (92). They are amphipatic molecules consisting of hydrophobic fatty acid chains (forming the bilayer of the membrane) esterified to a glycerol backbone (or sphingoside in sphingolipids) and a polar head group pointing towards the aequous phase.

The hydrophobic fatty acid chains of phospholipids vary in length. They are synthesized intracellularly by fatty acid synthase (FAS). Originating from malonyl-CoA, C2-units are added to an elongating fatty acid chain, and therefore hydrocarbon backbones of fatty acids usually are of even number of 16-20 carbon atoms. They may be saturated, meaning that all C-C bonds are single bonds or unsaturated with C=C double bonds occuring within the fatty acid chain, resulting in higher fluidity of membranes (93).

Whereas the fatty acid chains mainly change the physical properties of a membrane, the various polar head groups are of great importance for intracellular signaling. Many different headgroups are known, such as choline (resulting in phosphatidylcholine), ethanolamine (phosphatidylethanolamine), serine (phosphatidylserine) or inositol (phosphatidylinositol). For signaling, these headgroups serve as binding platforms that allow proteins to form complexes and excert their function at the appropriate intracellular locus (92).



Lipid	Relative level (%)	Fold increase on stimulation
Phosphatidylserine	8.5	1
Phosphatidic acid	1.5	1
Phosphatidylinositol	1.0	1
PtdIns(3)P	0.002	1
PtdIns(4)P	0.05	0.7
PtdIns(5)P	0.002	3-20
PtdIns(4,5)P_2	0.05	0.7
PtdIns(3,4)P_2	0.0001	10
PtdIns(3,5)P_2	0.0001	2-30

Fig. 6. Intracellular levels of phosphatidylinositides. Phosphatidylinositol (structure from (94)) can be phosphorylated at the 3-, 4- or 5- position. The relative intracellular amounts in erythrocytes are shown in table derived from (92). Phosphatidylinositol, PtdIns(4)P and PtdIns(4,5)P_2 show the highest relative amounts and are not induced upon stimulation, because they serve as precursors for inducible phosphatidylinositol phosphates. PtdIns(3,4,5)P_3 is barely detectable under unstimulated conditions and therefore does not appear on the list. When stimulated, however, it reaches levels comparable to PtdIns(3)P.

4.7.1 Phosphatidylinositides

Phosphatidylinositides consist of a diacyl-glycerol backbone with a D-*myo*-inositol headgroup that can be phosphorylated at the 3-, 4- or 5- position of the *myo*-inositol ring in living organisms (Fig. 6). Phosphatidylinositides play important roles in signaling and trafficking, serving as intracellular localisation markers for proteins, and orchestrate functional processes at the appropriate membrane structure (94). Thus, differentially phosphorylated phosphatidylinositides show different occurrence at different subcellular structures (Fig. 7), regulated by phospholipid-kinases and phosphatases. Due to the flexibility of phosphorylation at different residues, phosphatidylinositides can transduce signals very quickly, depending on the activities of the modifying kinases and phosphatases.

PtdIns and PtdIns(4)P

Phosphatidylinositol (PtdIns) is synthesized at the endoplasmic reticulum. Its precursor is phosphatidic acid. Phosphatidic acid has a very basic structure with a single phosphate as headgroup, and therefore serves as precursor of a number of phospholipids. In the endoplasmic reticulum, phosphatidic acid is converted to CDP-diacylglycerol by CDP-diacylglycerol synthetase. Subsequently, phosphatidylinositol synthase (CDP-diacylglycerol-inositol 3-phosphatidyltransferase) attaches a D-*myo*-inositol headgroup (95). Hence, PtdIns is predominantly found in the endoplasmic reticulum. PtdIns gets phosphorylated at the Golgi apparatus at the 4-position of the inositol headgroup. As mentionned above, changes in phosphorylation of phosphatidylinositides in cells are mediated by phosphatidylinositol kinases and phosphatases. Four kinases phosphorylating PtdIns at the 4-position are known, namely the type II kinases PI4KIIα and PI4KIIβ and the type III kinases PI4KIIIα and PI4KIIIβ (96). The major kinases of the Golgi apparatus are PI4KIIα (97-99) and PI4KIIIβ (100). The importance of PtdIns(4)P is substantiated by the phenotypes of cells disturbed in proper PtdIns(4)P synthesis. Overexpression of PI4KIIα increased the number of cells with either a scattered or no perinuclear Golgi apparatus, whereas knock-down of PI4KIIα resulted in a slightly expanded organelle (99). Also overexpression of PI4KIIIβ resulted in irregular perinuclear Golgi structures (100). Thus, PtdIns(4)P is found predominantly at the Golgi complex, and is indispensable for Golgi structure maintenance.

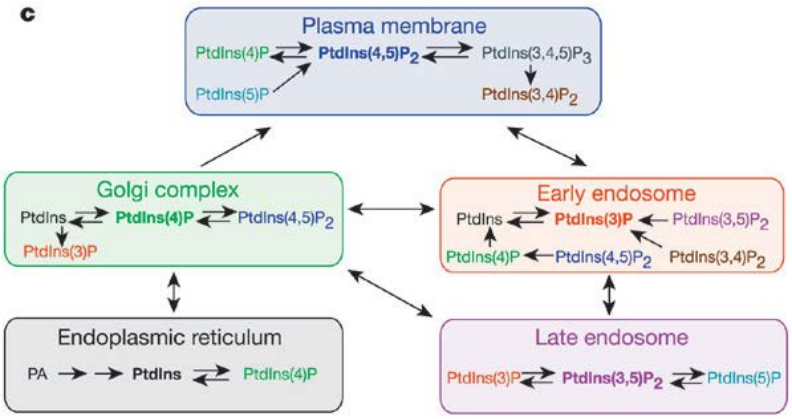


Fig. 7. Subcellular occurrence of phosphatidylinositides. Different phosphatidylinositides are localised at different membrane structures. Predominant species are PtdIns in the endoplasmic reticulum, PtdIns(4)P in the Golgi apparatus, PtdIns(4,5)P_2 at the plasma membrane, PtdIns(3)P in early endosomes and PtdIns(3,5)P_2 in late endosomes. See text for detailed explanations. (94)

PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3

Being one of the most abundant PtdIns species, PtdIns(4)P is phosphorylated by type I phosphatidylinositol 5-kinases to form PtdIns(4,5)P_2 (also known as PIP2), another very abundant phospholipid. The other way of synthesis, phosphorylation of PtdIns(5)P by type II PI4-kinases also occurs, but since PtdIns(5)P is not very abundant, this pathway is not discussed herein. Phosphorylation of PtdIns(4)P mainly occurs at the plasma membrane, as soon as PtdIns(4)P from the Golgi complex or recycling organelles reaches the plasma membrane. PtdIns(4,5)P_2 is involved in most processes at the plasma membrane, such as exocytosis, endocytosis, cell motility, signal transduction and cell adhesion (94).

PtdIns(4,5)P_2 is also well known as a substrate for intracellular signalling processes. Phospholipase C hydrolyses PtdIns(4,5)P_2, resulting in diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 serves as a second messenger that activates IP_3-receptors to release Ca^{2+} ions from the intracellular calcium storage in the endoplasmic reticulum into the cytoplasm (101). This IP_3 mediated Ca^{2+} signalling is of utmost importance in many physiological processes such as smooth muscle contraction (102) lymphocyte activation and T-cell proliferation (103) or proliferation of a fertilized ovum (104). The other second messenger derived from PtdIns(4,5)P_2 is DAG. This molecule is, together with Ca^{2+}, activating protein kinase C that signals through the MAP kinase pathway, regulating processes such as growth, differentiation or apoptosis (105).

For a different mode of signaling, PtdIns(4,5)P_2 serves as a substrate for phosphoinositide 3-kinases (PI 3-kinases). This conversion results in the rapid production of PtdIns(3,4,5)P_3 (or PIP3) at the plasma membrane. This is of major importance in a large number of signaling events that are mainly transduced by Akt-activation through phosphoinositide-dependent

kinase 1 (PDK-1) (50). The shut-down of the PtdIns(3,4,5)P_3-signal at the cell membrane is predominantly mediated by PTEN, a PI 3-phosphatase (106).

PtdIns(3)P and PtdIns(3,5)P_2

Phosphatidylinositides play an important role in endocytosis and endosomal trafficking. Internalisation of receptors in the clathrin coated vesicle pathway relies largely on PtdIns(4,5)P_2 which bind the clathrin adaptors AP-2 and AP180 (107-108). Upon internalisation, the vesicles need to be introduced into the endocytic system, which involves a set of lipid phosphatases and kinases. However, endosomes are identified by PtdIns(3)P as major phospholipid. The conversion of endocytosed membranes from outer cell membranes to PtdIns(3)P containing endosomes is not completely understood. One model suggests the production of a pool of PtdIns(3)P generated by lipid dephosphorylation of PtdIns(3,4,5)P_3 at the 5- and 4-position (109). A more sophisticated view, however, suggests lipid phosphatase and kinase activities dephosphorylating PtdIns(4,5)P_2 to PtdIns and subsequent phosphorylation at the 3-position. The importance of a 5-phosphatase activity in endocytosis has been nicely demonstrated for synaptic vesicle endocytosis at nerve terminals mediated by synaptojanin1 (110). Synaptojanin 1 is a dual phosphatase. It contains a 5-phosphatase activity that converts PtdIns(4,5)P_2 to PtdIns(4)P and a Sac1-like domain that acts less specifically by hydrolysing PtdIns(3,5)P_2, PtdIns(4)P and PtdIns(3)P to PtdIns (111). Cortical neurons of synaptojanin knock-out mice show an accumulation of clathrin coated vesicles within nerve terminals, suggesting that synaptojanin function is coupled to clathrin coat disassembly (110). For efficient endocytosis and clathrin coat maintenance, however, an equilibrium between PtdIns(4,5)P_2, PtdIns(4)P and PtdIns is required. To maintain this equilibrium, the PI 5-kinase PIPK Iγ counteracts dephosphorylation of synaptojanin 1 (112). For the final endocytic pool of PtdIns(3)P, the class III PI 3-kinase VPS34 is accounted for PtdIns phosphorylation (113).

In the endosomal compartment, PtdIns(3)P exerts its role by serving as a binding partner for a number of proteins involved in sorting. One prominent adaptor protein is the early-endosomal antigen 1 (EEA1) that possesses a binding domain for PtdIns(3)P (114). EEA1 interacts with SNARE proteins, which are important for membrane fusion throughout the endosomal system (115). Besides membrane fusion, endosomal trafficking along actin filaments and microtubules also depends on phospholipids. Kif16B represents such a kinesin motor that directly binds to PtdIns(3)P, executing early endosome motility (116).

Another phosphatidylionsitol species, PtdIns(3,5)P_2, is a very important lipid for the endosome-lysosome transition. This becomes clear by a vastly enlarged cellular vacuole when PIKfyve, the lipid kinase phosphorylating PtdIns(3)P at the 5-position of the inositol, is

inhibited (117). Effectors binding to PtdIns(3,5)P_2 are suggested to be involved in processes such as vacuolar membrane trafficking, vacuole acidification, stress-signaling and autophagy (118).

4.7.2 Phosphatidylinositide binding motifs

Phospholipids usually consist of acidic headgroups which are linked to the backbone as phosphoesters resulting in a negative charge pointing to the aequous phase. Thus, a hallmark of phospholipid binding is the presence of basic amino acid residues in the phospholipid binding region. For phosphatidic acid and phosphatidylserine, binding regions may consist of unstructured stretches of basic residues without displaying a known globular domain structure (119). The same holds true for phosphatidylinositides. However, for the differentially phosphorylated phosphatidylinositides with different subcellular localisations, lipid binding domains require a high degree of specificity for individual phosphatidylinositides, which is mainly mediated by well-defined and highly specific three dimensional folds (92).

Even though protein-lipid interactions are rather weak, binding strength can be increased by additional effects. For example hydrophobic structures in proximity to the basic residues may lead to partial integration of the protein into the lipid bilayer (120). Also oligomerisation of the lipid binding protein strengthens binding by increasing the avidity (i.e. number of binding sites for the oligomeric structure). This may even contribute to the binding specificity: if two proteins with lipid binding domains for different phospholipids oligomerise, a strong membrane localisation can only occur when both phospolipids are present in the target membrane (Fig.7). Furthermore, some domains require a membrane curvature, which is important for functions related to membrane budding in vesicle trafficking and directional movement of a cell (92).

Protein lipid overlay assays presented herein show binding of PASKIN to monophosphorylated phosphatidylinositides. For this reason, the following paragraphs will focus on some of the best characterised folds for phosphatidylinositide binding. All of the domains presented all bind monophosphorylated phosphatidylinositides. Further less-characterised domains or domains with other substrate specificities are not discussed herein and the reader is referred to excellent reviews (121,92).

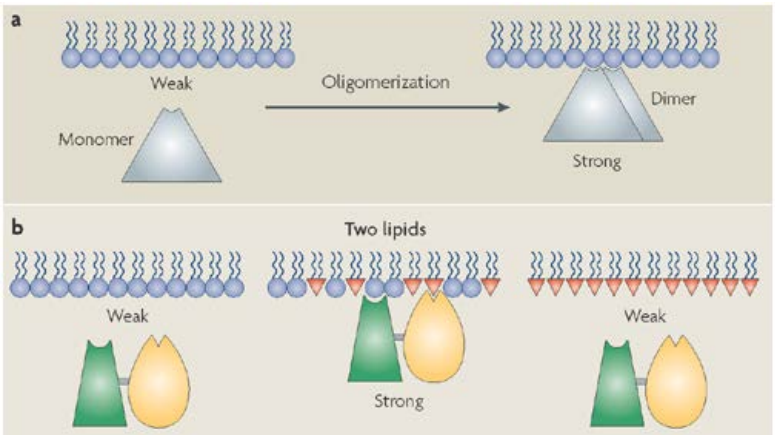


Fig. 7. Strength and specificity of phospholipid binding. (a) Phospholipid binding can be strengthened by oligomerisation. (b) If oligomers show specificity for different phospholipids, the specificity for target membranes consisting of both phospholipids is increased and allows spatially regulated protein activity. (92)

Pleckstrin homology (PH) domains

The pleckstrin homology (PH) domain is the oldest phosphatidylinositide binding domain described. In the platelet protein pleckstrin, two such domains bind to PtdIns(4,5)P_2 (122). The pleckstin homology domain is characterised by a region of ~100 amino acids of sequence homology. Structurally, the canonical pleckstrin homology domain consists of a seven stranded β-sandwich with a C-terminal α-helix. The loop between β1 and β2 displays the sequence motif KX_n(K/R)XR, where the basic amino acids form substrate residues for the headgroup-phosphate binding of the lipid. PH domains are the most frequent binding modules found in more than 250 proteins (92). Many examples for PH domains are known to bind PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 with high specificity (92). To outline the high specificity, it was shown that PtdIns(3,4,5)P_3 binding PH domains formed 17-19 hydrogen bonds between lipid phosphates and spatially distributed basic headgroups in co-crystallisation experiments (123).

In addition to these canonical PH domains, so called non-canonical PH domains have been identified in TIAM1 and Rho GTPase-activating protein-9 (ARHGAP9) (124). Analysis of the sequence homology and structure suggest the involvement of two pairs of basic residues (R or K) in the β1-β2 loop and in the β5-β6 loop instead of the canonical KX_n(K/R)XR motif. The involvement of two double lysine (KK) motifs has also been reported to mediate PtdIns(4,5)P_2 binding for Toll-interleukin1 receptor domain containing adaptor protein (TIRAP) (125), a scaffolding protein for Toll-like receptor 2 and 4 signaling. There exist a number of examples where PH-domains are binding to PtdIns(4)P. This was described for four-phosphate-adaptor proteins (FAPP) 1 and 2, that are important for membrane trafficking from the trans-Golgi network to the cell surface membrane (126). An interesting example of a non-canonical PH-domain with a broad range of phosphatidylinositide binding is present in

the yeast Vps36 (ESCRT-II) protein. Liposome sedimentation assays showed substantial binding to PtdIns(3)P and weaker binding to PtdIns(4)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (127). Since ESCRT complexes are the essential proteins for endosome-lysosome sorting, preferential binding of Vps36 to endosomal PtdIns(3)P is substantial for appropriate function (127).

PX domains

PX domains are of ~130 amino acids length and are named after the phagocyte NADPH oxidase (phox) complex. The binding pocket of a PX-domain consists of an N-terminal three stranded β-sheet and an α-helical domain. In the $p40^{phox}$ subunit, binding of PtdIns occurs via hydrogen bonds to conserved basic headgroups in the pocket between the β-sheet and the α-helical subdomain (128). From NMR structural analysis of Vam7 (129), it has been suggested that PX domains display partial insertion of an α-helical structure into the lipid bilayer which essentially contributes to lipid binding. They show specificity for monophosphorylated phosphatidylinositides because additional phosphate groups are sterically infavourable (128). In line with the common specificity for PtdIns(3)P, which mainly occurs in endosomal membranes, many PX domain proteins are involved in vesicular trafficking and protein sorting (130). However, binding of PX domains to other phosphoinositides has been reported as well, for example NOXO1 (nox organising protein 1) binds to PtdIns(4)P, PtdIns(5)P and PtdIns(3,5)P_2 (131).

FYVE domains

FYVE is an acronym for four proteins in which such a domain was identified, namely Fab1, YOTB, Vac1 and EEA1 (132). It is a 60-70 amino acids zinc-finger domain containing two β-hairpins. In the first β-strand, FYVE proteins contain a RR/KHHCR basic motif that binds to PtdIns(3)P (114). Characteristic of FYVE domains is their much stronger binding to membrane-embedded PtdIns(3)P because of partial membrane insertion of the domain and oligomerisation of the protein. The binding mechanism has been nicely demonstrated for the early endosome antigen 1 (EEA1). High resolution NMR and X-ray structures revealed the precise residues forming the hydrogen bonds with the lipid, membrane insertion and the increased avidity of PtdIns(3)P binding by dimerisation (133-134). Another FYVE-domain containing protein is the lipid kinase PIKfyve. Its FYVE-domain binds PtdIns(3)P, and subsequently PIKfyve phosphorylates phosphatidylinositides at the 5'-position generating PtdIns(3,5)P_2 and PtdIns(5)P (135). The FYVE-domain thus ensures the appropriate subcellular localisation at the endosomes, where PIKfyve can trigger the endosome-lysosome transition by generating PtdIns(3,5)P_2. The importance of this mechanism was nicely demonstrated by overexpression of a dominant negative kinase-deficient K1831E mutant that

displayed exacerbated cytoplasmic vacuolation (117), implying a crucial function in endomembrane homeostasis.

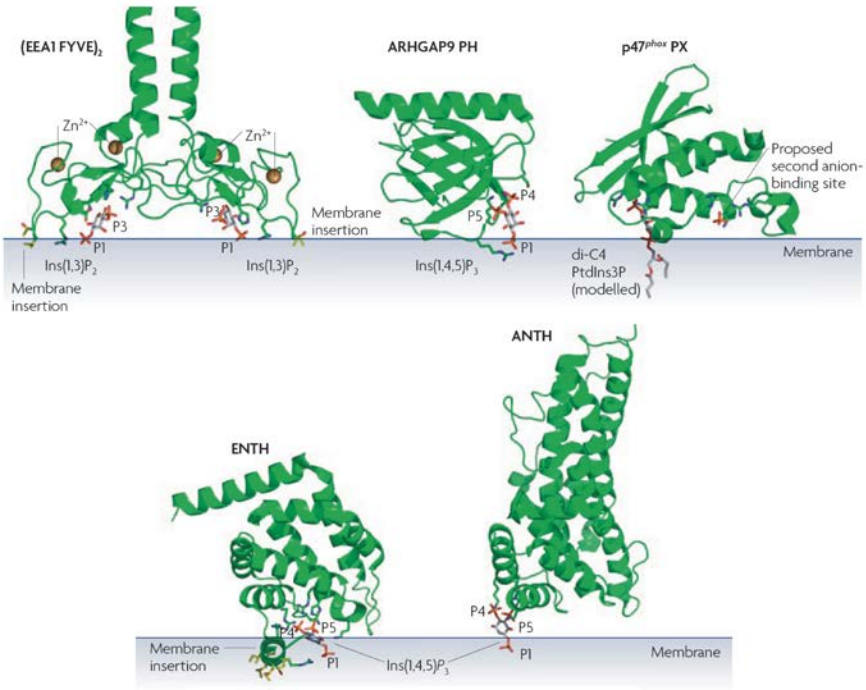


Fig. 8. Phospholipid binding domains. The dimer of the FYVE domain of EEA1 (PDB: 1JOC) binds to PtdIns(3)P and shows membrane insertion. The non-canonical PH domain of ARHGAP9 (2P0D) binds to PtdIns(4,5)P_2. The PX domain of $p47^{phox}$ is supposed to bind PtdIns(3)P (1O7K) and has a proposed second anin-binding site presumably binding phosphatidic acid. The PtdIns(4,5)P_2 binding ANTH domain of AP 180 (1HFA) does not show the additional helix 0 of the ENTH domain of epsin (1H0A). The amphipathic helix 0 is inserted into the membrane and deforms the lipid bilayer priming vesicle budding. (Fig. modified from (92))

ENTH (ANTH) domains

Two domains with homologous structure derive from proteins involved in clathrin-mediated endocytosis and cytoskeleton organisation. These domains are called epsin NH_2-terminal homology (ENTH) and AP180 NH_2-terminal homology (ANTH) domain. In epsin1, the ENTH domain primarily binds PtdIns(4,5)P_2 (136). However, the related EpsinR prefers PtdIns(4)P and PtdIns(5)P, rather than PtdIns(4,5)P_2. Structural analysis could demonstrate that residues involved in PtdIns(4,5)P_2 binding in epsin1 were altered in epsinR despite a high overall sequence identity of 48% between the two ENTH domains (137). As expected, PtdIns(4,5)P_2 binding epsin1 is involved in clathrin-coated vesicle budding at the cell surface membrane, whereas PtdIns(4)P binding epsinR has the identical function in clathrin-coated vesicle budding in the *trans*-Golgi network and endosomal compartments (137). Noteworthy, ENTH domains insert an α-helix (called helix 0) into their target membrane, and thus deform the membrane. This deformation primes the membrane invagination needed for clathrin-coated vesicle formation (138).

4.8 Goals of the thesis

First, we aimed to identify and characterise novel phosphorylation targets of PASKIN. Based on the findings in yeast and the physiological function in energy homeostasis in mammals, we were particularly interested in targets involved in protein translation and metabolism. The first target we characterised was eEF1A1. Interestingly, PASKIN phosphorylates eEF1A1 at the same site as published for PKC. Since PKC needs phospholipid co-activators for full kinase function, we addressed the second question of the thesis; whether phospholipids or their headgroups could be the long sought-for activating ligands of the PAS domain of PASKIN modifiying kinase function. Finally, we were interested to confirm the phenotype published for *Paskin*$^{-/-}$ male mice upon feeding of a HFD. Accordingly, *Paskin*$^{-/-}$ males should be partially protected from HFD induced obesity and insulin resistance. Based on our results, we intended to clarify potential mechanistics underlying this phenotype.

4.9 References

1 Hoffman EC, Reyes H, Chu FF, Sander F, Conley LH, Brooks BA, Hankinson O (1991) Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* **252**: 954-958.

2 Lowrey PL, Takahashi JS (2000) Genetics of the mammalian circadian system: Photic entrainment, circadian pacemaker mechanisms, and posttranslational regulation. *Annu Rev Genet* **34**: 533-562.

3 Mimura J, Fujii-Kuriyama Y (2003) Functional role of AhR in the expression of toxic effects by TCDD. *Biochim Biophys Acta* **1619**: 263-268.

4 Wenger RH (2002) Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. *Faseb J* **16**: 1151-1162.

5 Chen H, Chrast R, Rossier C, Gos A, Antonarakis SE, Kudoh J, Yamaki A, Shindoh N, Maeda H, Minoshima S, et al. (1995) Single-minded and Down syndrome? *Nat Genet* **10**: 9-10.

6 Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R, Eddy SR, Sonnhammer EL, Bateman A (2006) Pfam: clans, web tools and services. *Nucleic Acids Res* **34**: D247-251.

7 Schlafli P, Borter E, Spielmann P, Wenger RH (2009) The PAS-domain kinase PASKIN: a new sensor in energy homeostasis. *Cell Mol Life Sci* **66**: 876-883.

8 Lendahl U, Lee KL, Yang H, Poellinger L (2009) Generating specificity and diversity in the transcriptional response to hypoxia. *Nat Rev Genet* **10**: 821-832.

9 Moglich A, Ayers RA, Moffat K (2009) Structure and signaling mechanism of Per-ARNT-Sim domains. *Structure* **17**: 1282-1294.

10 Dioum EM, Rutter J, Tuckerman JR, Gonzalez G, Gilles-Gonzalez MA, McKnight SL (2002) NPAS2: a gas-responsive transcription factor. *Science* **298**: 2385-2387.

11 Boehning D, Snyder SH (2002) Circadian rhythms. Carbon monoxide and clocks. *Science* **298**: 2339-2340.

12 Crosson S, Moffat K (2001) Structure of a flavin-binding plant photoreceptor domain: insights into light-mediated signal transduction. *Proc Natl Acad Sci U S A* **98**: 2995-3000.

13 Christie JM, Reymond P, Powell GK, Bernasconi P, Raibekas AA, Liscum E, Briggs WR (1998) Arabidopsis NPH1: a flavoprotein with the properties of a photoreceptor for phototropism. *Science* **282**: 1698-1701.

14 Morais Cabral JH, Lee A, Cohen SL, Chait BT, Li M, Mackinnon R (1998) Crystal structure and functional analysis of the HERG potassium channel N terminus: a eukaryotic PAS domain. *Cell* **95**: 649-655.

15 Taylor BL, Zhulin IB (1999) PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol Mol Biol Rev* **63**: 479-506.

16 Card PB, Erbel PJ, Gardner KH (2005) Structural basis of ARNT PAS-B dimerization: use of a common beta-sheet interface for hetero- and homodimerization. *J Mol Biol* **353**: 664-677.

17 Amezcua CA, Harper SM, Rutter J, Gardner KH (2002) Structure and interactions of PAS kinase N-terminal PAS domain: model for intramolecular kinase regulation. *Structure* **10**: 1349-1361.

18 Miyatake H, Mukai M, Park SY, Adachi S, Tamura K, Nakamura H, Nakamura K, Tsuchiya T, Iizuka T, Shiro Y (2000) Sensory mechanism of oxygen sensor FixL from Rhizobium meliloti: crystallographic, mutagenesis and resonance Raman spectroscopic studies. *J Mol Biol* **301**: 415-431.

19 Sousa EH, Tuckerman JR, Gonzalez G, Gilles-Gonzalez MA (2007) A memory of oxygen binding explains the dose response of the heme-based sensor FixL. *Biochemistry* **46**: 6249-6257.

20 Hofer T, Spielmann P, Stengel P, Stier B, Katschinski DM, Desbaillets I, Gassmann M, Wenger RH (2001) Mammalian PASKIN, a PAS-serine/threonine kinase related to bacterial oxygen sensors. *Biochem Biophys Res Commun* **288**: 757-764.

21 Rutter J, Michnoff CH, Harper SM, Gardner KH, McKnight SL (2001) PAS kinase: an evolutionarily conserved PAS domain-regulated serine/threonine kinase. *Proc Natl Acad Sci U S A* **98**: 8991-8996.

22 Ushiro H, Tsutsumi T, Suzuki K, Kayahara T, Nakano K (1998) Molecular cloning and characterization of a novel Ste20-related protein kinase enriched in neurons and transporting epithelia. *Arch Biochem Biophys* **355**: 233-240.

23 Katschinski DM, Marti HH, Wagner KF, Shibata J, Eckhardt K, Martin F, Depping R, Paasch U, Gassmann M, Ledermann B, Desbaillets I, Wenger RH (2003) Targeted disruption of the mouse PAS domain serine/threonine kinase PASKIN. *Mol Cell Biol* **23**: 6780-6789.

24 Borter E, Niessen M, Zuellig R, Spinas GA, Spielmann P, Camenisch G, Wenger RH (2007) Glucose-stimulated insulin production in mice deficient for the PAS kinase PASKIN. *Diabetes* **56**: 113-117.

25 Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. *Science* **298**: 1912-1934.

26 Kikani CK, Antonysamy SA, Bonanno JB, Romero R, Zhang FF, Russell M, Gheyi T, Iizuka M, Emtage S, Sauder JM, Turk BE, Burley SK, Rutter J (2010) Structural bases of PAS domain-regulated kinase (PASK) activation in the absence of activation loop phosphorylation. *J Biol Chem* **285**: 41034-41043.

27 Nolen B, Taylor S, Ghosh G (2004) Regulation of protein kinases; controlling activity through activation segment conformation. *Mol Cell* **15**: 661-675.

28 Rutter J, Probst BL, McKnight SL (2002) Coordinate regulation of sugar flux and translation by PAS kinase. *Cell* **111**: 17-28.

29 Wilson WA, Skurat AV, Probst B, de Paoli-Roach A, Roach PJ, Rutter J (2005) Control of mammalian glycogen synthase by PAS kinase. *Proc Natl Acad Sci U S A* **102**: 16596-16601.

30 An R, da Silva Xavier G, Hao HX, Semplici F, Rutter J, Rutter GA (2006) Regulation by Per-Arnt-Sim (PAS) kinase of pancreatic duodenal homeobox-1 nuclear import in pancreatic beta-cells. *Biochem Soc Trans* **34**: 791-793.

31 Eckhardt K, Troger J, Reissmann J, Katschinski DM, Wagner KF, Stengel P, Paasch U, Hunziker P, Borter E, Barth S, Schlafli P, Spielmann P, Stiehl DP, Camenisch G, Wenger RH (2007) Male germ cell expression of the PAS domain kinase PASKIN and its novel target eukaryotic translation elongation factor eEF1A1. *Cell Physiol Biochem* **20**: 227-240.

32 Grose JH, Sundwall E, Rutter J (2009) Regulation and function of yeast PAS kinase: a role in the maintenance of cellular integrity. *Cell Cycle* **8**: 1824-1832.

33 Smith TL, Rutter J (2007) Regulation of glucose partitioning by PAS kinase and Ugp1 phosphorylation. *Mol Cell* **26**: 491-499.

34 Rauceo JM, Blankenship JR, Fanning S, Hamaker JJ, Deneault JS, Smith FJ, Nantel A, Mitchell AP (2008) Regulation of the Candida albicans cell wall damage response by transcription factor Sko1 and PAS kinase Psk1. *Mol Biol Cell* **19**: 2741-2751.

35 IDF (2009) IDF Diabetes Atlas. 4th edn. International Diabetes Federation, Brussels

36 Elrick LJ, Docherty K (2001) Phosphorylation-dependent nucleocytoplasmic shuttling of pancreatic duodenal homeobox-1. *Diabetes* **50**: 2244-2252.

37 da Silva Xavier G, Rutter J, Rutter GA (2004) Involvement of Per-Arnt-Sim (PAS) kinase in the stimulation of preproinsulin and pancreatic duodenum

homeobox 1 gene expression by glucose. *Proc Natl Acad Sci U S A* **101**: 8319-8324.

38 Prentki M, Joly E, El-Assaad W, Roduit R (2002) Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes. *Diabetes* **51 Suppl 3**: S405-413.

39 El-Assaad W, Buteau J, Peyot ML, Nolan C, Roduit R, Hardy S, Joly E, Dbaibo G, Rosenberg L, Prentki M (2003) Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology* **144**: 4154-4163.

40 Kelpe CL, Moore PC, Parazzoli SD, Wicksteed B, Rhodes CJ, Poitout V (2003) Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis. *J Biol Chem* **278**: 30015-30021.

41 Fontes G, Semache M, Hagman DK, Tremblay C, Shah R, Rhodes CJ, Rutter J, Poitout V (2009) Involvement of Per-Arnt-Sim Kinase and extracellular-regulated kinases-1/2 in palmitate inhibition of insulin gene expression in pancreatic beta-cells. *Diabetes* **58**: 2048-2058.

42 Hao HX, Cardon CM, Swiatek W, Cooksey RC, Smith TL, Wilde J, Boudina S, Abel ED, McClain DA, Rutter J (2007) PAS kinase is required for normal cellular energy balance. *Proc Natl Acad Sci U S A* **104**: 15466-15471.

43 Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* **10**: 457-468.

44 Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol* **6**: 1122-1128.

45 Proud CG (2007) Amino acids and mTOR signalling in anabolic function. *Biochem Soc Trans* **35**: 1187-1190.

46 Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM (2002) mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**: 163-175.

47 Inoki K, Zhu T, Guan KL (2003) TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115**: 577-590.

48 Wullschleger S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. *Cell* **124**: 471-484.

49 Backer JM, Myers MG, Jr., Shoelson SE, Chin DJ, Sun XJ, Miralpeix M, Hu P, Margolis B, Skolnik EY, Schlessinger J, et al. (1992) Phosphatidylinositol

3'-kinase is activated by association with IRS-1 during insulin stimulation. *Embo J* **11**: 3469-3479.

50 Vanhaesebroeck B, Alessi DR (2000) The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J* **346 Pt 3**: 561-576.

51 Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC (2002) Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol Cell* **10**: 151-162.

52 Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J, Thomas G (2004) Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* **431**: 200-205.

53 Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE (2001) Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108**: 1167-1174.

54 Meyuhas O (2008) Physiological roles of ribosomal protein S6: one of its kind. *Int Rev Cell Mol Biol* **268**: 1-37.

55 Sonenberg N, Hinnebusch AG (2009) Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* **136**: 731-745.

56 Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K (2002) Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* **110**: 177-189.

57 Zanchin NI, McCarthy JE (1995) Characterization of the in vivo phosphorylation sites of the mRNA.cap-binding complex proteins eukaryotic initiation factor-4E and p20 in Saccharomyces cerevisiae. *J Biol Chem* **270**: 26505-26510.

58 de la Cruz J, Iost I, Kressler D, Linder P (1997) The p20 and Ded1 proteins have antagonistic roles in eIF4E-dependent translation in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **94**: 5201-5206.

59 Chaudhuri J, Si K, Maitra U (1997) Function of eukaryotic translation initiation factor 1A (eIF1A) (formerly called eIF-4C) in initiation of protein synthesis. *J Biol Chem* **272**: 7883-7891.

60 Sobel SG, Wolin SL (1999) Two yeast La motif-containing proteins are RNA-binding proteins that associate with polyribosomes. *Mol Biol Cell* **10**: 3849-3862.

61 Rother S, Burkert C, Brunger KM, Mayer A, Kieser A, Strasser K (2010) Nucleocytoplasmic shuttling of the La motif-containing protein Sro9 might link its nuclear and cytoplasmic functions. *Rna* **16**: 1393-1401.

62 Albuquerque CP, Smolka MB, Payne SH, Bafna V, Eng J, Zhou H (2008) A multidimensional chromatography technology for in-depth phosphoproteome analysis. *Mol Cell Proteomics* **7**: 1389-1396.

63 Soares DC, Barlow PN, Newbery HJ, Porteous DJ, Abbott CM (2009) Structural models of human eEF1A1 and eEF1A2 reveal two distinct surface clusters of sequence variation and potential differences in phosphorylation. *PLoS One* **4**: e6315.

64 Lee S, Francoeur AM, Liu S, Wang E (1992) Tissue-specific expression in mammalian brain, heart, and muscle of S1, a member of the elongation factor-1 alpha gene family. *J Biol Chem* **267**: 24064-24068.

65 Khalyfa A, Bourbeau D, Chen E, Petroulakis E, Pan J, Xu S, Wang E (2001) Characterization of elongation factor-1A (eEF1A-1) and eEF1A-2/S1 protein expression in normal and wasted mice. *J Biol Chem* **276**: 22915-22922.

66 Stapulionis R, Kolli S, Deutscher MP (1997) Efficient mammalian protein synthesis requires an intact F-actin system. *J Biol Chem* **272**: 24980-24986.

67 Liu G, Grant WM, Persky D, Latham VM, Jr., Singer RH, Condeelis J (2002) Interactions of elongation factor 1alpha with F-actin and beta-actin mRNA: implications for anchoring mRNA in cell protrusions. *Mol Biol Cell* **13**: 579-592.

68 Guo A, Villen J, Kornhauser J, Lee KA, Stokes MP, Rikova K, Possemato A, Nardone J, Innocenti G, Wetzel R, Wang Y, MacNeill J, Mitchell J, Gygi SP, Rush J, Polakiewicz RD, Comb MJ (2008) Signaling networks assembled by oncogenic EGFR and c-Met. *Proc Natl Acad Sci U S A* **105**: 692-697.

69 Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, Nardone J, Lee K, Reeves C, Li Y, Hu Y, Tan Z, Stokes M, Sullivan L, Mitchell J, Wetzel R, Macneill J, Ren JM, Yuan J, Bakalarski CE, Villen J, Kornhauser JM, Smith B, Li D, Zhou X, Gygi SP, Gu TL, Polakiewicz RD, Rush J, Comb MJ (2007) Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* **131**: 1190-1203.

70 Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, Zhang H, Zha XM, Polakiewicz RD, Comb MJ (2005) Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat Biotechnol* **23**: 94-101.

71 Kielbassa K, Muller HJ, Meyer HE, Marks F, Gschwendt M (1995) Protein kinase C delta-specific phosphorylation of the elongation factor eEF-alpha and an eEF-1 alpha peptide at threonine 431. *J Biol Chem* **270**: 6156-6162.

72 Venema RC, Peters HI, Traugh JA (1991) Phosphorylation of elongation factor 1 (EF-1) and valyl-tRNA synthetase by protein kinase C and stimulation of EF-1 activity. *J Biol Chem* **266**: 12574-12580.

73 Peters HI, Chang YW, Traugh JA (1995) Phosphorylation of elongation factor 1 (EF-1) by protein kinase C stimulates GDP/GTP-exchange activity. *Eur J Biochem* **234**: 550-556.

74 Izawa T, Fukata Y, Kimura T, Iwamatsu A, Dohi K, Kaibuchi K (2000) Elongation factor-1 alpha is a novel substrate of rho-associated kinase. *Biochem Biophys Res Commun* **278**: 72-78.

75 Lamberti A, Longo O, Marra M, Tagliaferri P, Bismuto E, Fiengo A, Viscomi C, Budillon A, Rapp UR, Wang E, Venuta S, Abbruzzese A, Arcari P, Caraglia M (2007) C-Raf antagonizes apoptosis induced by IFN-alpha in human lung cancer cells by phosphorylation and increase of the intracellular content of elongation factor 1A. *Cell Death Differ* **14**: 952-962.

76 Kabat D (1970) Phosphorylation of ribosomal proteins in rabbit reticulocytes. Characterization and regulatory aspects. *Biochemistry* **9**: 4160.

77 Ferrari S, Bandi HR, Hofsteenge J, Bussian BM, Thomas G (1991) Mitogen-activated 70K S6 kinase. Identification of in vitro 40 S ribosomal S6 phosphorylation sites. *J Biol Chem* **266**: 22770-22775.

78 Brown EJ, Beal PA, Keith CT, Chen J, Shin TB, Schreiber SL (1995) Control of p70 s6 kinase by kinase activity of FRAP in vivo. *Nature* **377**: 441-446.

79 Roux PP, Shahbazian D, Vu H, Holz MK, Cohen MS, Taunton J, Sonenberg N, Blenis J (2007) RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *J Biol Chem* **282**: 14056-14064.

80 Andres JL, Johansen JW, Maller JL (1987) Identification of protein phosphatases 1 and 2B as ribosomal protein S6 phosphatases in vitro and in vivo. *J Biol Chem* **262**: 14389-14393.

81 Meyuhas O, Avni D, Shama S (1996) Translational Control of Ribosomal Protein mRNAs in Eukaryotes. In: Hershey JWB, Matthews MB, Sonenberg N (eds) Translational Control. Cold Spring Harbor Laboratory Press, pp 363-388

82 Loreni F, Francesconi A, Amaldi F (1993) Coordinate translational regulation in the syntheses of elongation factor 1 alpha and ribosomal proteins in Xenopus laevis. *Nucleic Acids Res* **21**: 4721-4725.

83 Iadevaia V, Caldarola S, Tino E, Amaldi F, Loreni F (2008) All translation elongation factors and the e, f, and h subunits of translation initiation factor 3 are encoded by 5'-terminal oligopyrimidine (TOP) mRNAs. *Rna* **14**: 1730-1736.

84 Jefferies HB, Reinhard C, Kozma SC, Thomas G (1994) Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. *Proc Natl Acad Sci U S A* **91**: 4441-4445.

85 Volarevic S, Stewart MJ, Ledermann B, Zilberman F, Terracciano L, Montini E, Grompe M, Kozma SC, Thomas G (2000) Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. *Science* **288**: 2045-2047.

86 Sulic S, Panic L, Barkic M, Mercep M, Uzelac M, Volarevic S (2005) Inactivation of S6 ribosomal protein gene in T lymphocytes activates a p53-dependent checkpoint response. *Genes Dev* **19**: 3070-3082.

87 Shima H, Pende M, Chen Y, Fumagalli S, Thomas G, Kozma SC (1998) Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. *Embo J* **17**: 6649-6659.

88 Pende M, Um SH, Mieulet V, Sticker M, Goss VL, Mestan J, Mueller M, Fumagalli S, Kozma SC, Thomas G (2004) S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol* **24**: 3112-3124.

89 Ruvinsky I, Sharon N, Lerer T, Cohen H, Stolovich-Rain M, Nir T, Dor Y, Zisman P, Meyuhas O (2005) Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev* **19**: 2199-2211.

90 Ruvinsky I, Katz M, Dreazen A, Gielchinsky Y, Saada A, Freedman N, Mishani E, Zimmerman G, Kasir J, Meyuhas O (2009) Mice deficient in ribosomal protein S6 phosphorylation suffer from muscle weakness that reflects a growth defect and energy deficit. *PLoS One* **4**: e5618.

91 Soliz J, Soulage C, Borter E, van Patot MT, Gassmann M (2008) Ventilatory responses to acute and chronic hypoxia are altered in female but not male Paskin-deficient mice. *Am J Physiol Regul Integr Comp Physiol* **295**: R649-658.

92 Lemmon MA (2008) Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol* **9**: 99-111.

93 Spector AA, Yorek MA (1985) Membrane lipid composition and cellular function. *J Lipid Res* **26**: 1015-1035.

94 Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**: 651-657.

95 Lykidis A, Jackson PD, Rock CO, Jackowski S (1997) The role of CDP-diacylglycerol synthetase and phosphatidylinositol synthase activity levels in

the regulation of cellular phosphatidylinositol content. *J Biol Chem* **272**: 33402-33409.

96 Balla A, Balla T (2006) Phosphatidylinositol 4-kinases: old enzymes with emerging functions. *Trends Cell Biol* **16**: 351-361.

97 Minogue S, Anderson JS, Waugh MG, dos Santos M, Corless S, Cramer R, Hsuan JJ (2001) Cloning of a human type II phosphatidylinositol 4-kinase reveals a novel lipid kinase family. *J Biol Chem* **276**: 16635-16640.

98 Barylko B, Gerber SH, Binns DD, Grichine N, Khvotchev M, Sudhof TC, Albanesi JP (2001) A novel family of phosphatidylinositol 4-kinases conserved from yeast to humans. *J Biol Chem* **276**: 7705-7708.

99 Wang YJ, Wang J, Sun HQ, Martinez M, Sun YX, Macia E, Kirchhausen T, Albanesi JP, Roth MG, Yin HL (2003) Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. *Cell* **114**: 299-310.

100 Godi A, Pertile P, Meyers R, Marra P, Di Tullio G, Iurisci C, Luini A, Corda D, De Matteis MA (1999) ARF mediates recruitment of PtdIns-4-OH kinase-beta and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex. *Nat Cell Biol* **1**: 280-287.

101 Berridge MJ (2009) Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta* **1793**: 933-940.

102 Abdel-Latif AA (2001) Cross talk between cyclic nucleotides and polyphosphoinositide hydrolysis, protein kinases, and contraction in smooth muscle. *Exp Biol Med (Maywood)* **226**: 153-163.

103 Lewis RS (2001) Calcium signaling mechanisms in T lymphocytes. *Annu Rev Immunol* **19**: 497-521.

104 Ajduk A, Malagocki A, Maleszewski M (2008) Cytoplasmic maturation of mammalian oocytes: development of a mechanism responsible for sperm-induced Ca2+ oscillations. *Reprod Biol* **8**: 3-22.

105 Nishizuka Y (1986) Studies and perspectives of protein kinase C. *Science* **233**: 305-312.

106 Maehama T, Dixon JE (1999) PTEN: a tumour suppressor that functions as a phospholipid phosphatase. *Trends Cell Biol* **9**: 125-128.

107 Rohde G, Wenzel D, Haucke V (2002) A phosphatidylinositol (4,5)-bisphosphate binding site within mu2-adaptin regulates clathrin-mediated endocytosis. *J Cell Biol* **158**: 209-214.

108 Ford MG, Pearse BM, Higgins MK, Vallis Y, Owen DJ, Gibson A, Hopkins CR, Evans PR, McMahon HT (2001) Simultaneous binding of PtdIns(4,5)P2

and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science* **291**: 1051-1055.

109 Shin HW, Hayashi M, Christoforidis S, Lacas-Gervais S, Hoepfner S, Wenk MR, Modregger J, Uttenweiler-Joseph S, Wilm M, Nystuen A, Frankel WN, Solimena M, De Camilli P, Zerial M (2005) An enzymatic cascade of Rab5 effectors regulates phosphoinositide turnover in the endocytic pathway. *J Cell Biol* **170**: 607-618.

110 Cremona O, Di Paolo G, Wenk MR, Luthi A, Kim WT, Takei K, Daniell L, Nemoto Y, Shears SB, Flavell RA, McCormick DA, De Camilli P (1999) Essential role of phosphoinositide metabolism in synaptic vesicle recycling. *Cell* **99**: 179-188.

111 Guo S, Stolz LE, Lemrow SM, York JD (1999) SAC1-like domains of yeast SAC1, INP52, and INP53 and of human synaptojanin encode polyphosphoinositide phosphatases. *J Biol Chem* **274**: 12990-12995.

112 Wenk MR, Pellegrini L, Klenchin VA, Di Paolo G, Chang S, Daniell L, Arioka M, Martin TF, De Camilli P (2001) PIP kinase Igamma is the major PI(4,5)P(2) synthesizing enzyme at the synapse. *Neuron* **32**: 79-88.

113 Christoforidis S, Miaczynska M, Ashman K, Wilm M, Zhao L, Yip SC, Waterfield MD, Backer JM, Zerial M (1999) Phosphatidylinositol-3-OH kinases are Rab5 effectors. *Nat Cell Biol* **1**: 249-252.

114 Simonsen A, Lippe R, Christoforidis S, Gaullier JM, Brech A, Callaghan J, Toh BH, Murphy C, Zerial M, Stenmark H (1998) EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature* **394**: 494-498.

115 McBride HM, Rybin V, Murphy C, Giner A, Teasdale R, Zerial M (1999) Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. *Cell* **98**: 377-386.

116 Hoepfner S, Severin F, Cabezas A, Habermann B, Runge A, Gillooly D, Stenmark H, Zerial M (2005) Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B. *Cell* **121**: 437-450.

117 Ikonomov OC, Sbrissa D, Shisheva A (2001) Mammalian cell morphology and endocytic membrane homeostasis require enzymatically active phosphoinositide 5-kinase PIKfyve. *J Biol Chem* **276**: 26141-26147.

118 Dove SK, Dong K, Kobayashi T, Williams FK, Michell RH (2009) Phosphatidylinositol 3,5-bisphosphate and Fab1p/PIKfyve underPPIn endo-lysosome function. *Biochem J* **419**: 1-13.

119 Stace CL, Ktistakis NT (2006) Phosphatidic acid- and phosphatidylserine-binding proteins. *Biochim Biophys Acta* **1761**: 913-926.

120 Lemmon MA (2003) Phosphoinositide recognition domains. *Traffic* **4**: 201-213.

121 Balla T (2005) Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. *J Cell Sci* **118**: 2093-2104.

122 Harlan JE, Hajduk PJ, Yoon HS, Fesik SW (1994) Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. *Nature* **371**: 168-170.

123 Ferguson KM, Kavran JM, Sankaran VG, Fournier E, Isakoff SJ, Skolnik EY, Lemmon MA (2000) Structural basis for discrimination of 3-phosphoinositides by pleckstrin homology domains. *Mol Cell* **6**: 373-384.

124 Ceccarelli DF, Blasutig IM, Goudreault M, Li Z, Ruston J, Pawson T, Sicheri F (2007) Non-canonical interaction of phosphoinositides with pleckstrin homology domains of Tiam1 and ArhGAP9. *J Biol Chem* **282**: 13864-13874.

125 Kagan JC, Medzhitov R (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* **125**: 943-955.

126 Godi A, Di Campli A, Konstantakopoulos A, Di Tullio G, Alessi DR, Kular GS, Daniele T, Marra P, Lucocq JM, De Matteis MA (2004) FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P. *Nat Cell Biol* **6**: 393-404.

127 Teo H, Gill DJ, Sun J, Perisic O, Veprintsev DB, Vallis Y, Emr SD, Williams RL (2006) ESCRT-I core and ESCRT-II GLUE domain structures reveal role for GLUE in linking to ESCRT-I and membranes. *Cell* **125**: 99-111.

128 Bravo J, Karathanassis D, Pacold CM, Pacold ME, Ellson CD, Anderson KE, Butler PJ, Lavenir I, Perisic O, Hawkins PT, Stephens L, Williams RL (2001) The crystal structure of the PX domain from p40(phox) bound to phosphatidylinositol 3-phosphate. *Mol Cell* **8**: 829-839.

129 Cheever ML, Sato TK, de Beer T, Kutateladze TG, Emr SD, Overduin M (2001) Phox domain interaction with PtdIns(3)P targets the Vam7 t-SNARE to vacuole membranes. *Nat Cell Biol* **3**: 613-618.

130 Lindmo K, Stenmark H (2006) Regulation of membrane traffic by phosphoinositide 3-kinases. *J Cell Sci* **119**: 605-614.

131 Cheng G, Lambeth JD (2004) NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain. *J Biol Chem* **279**: 4737-4742.

132 Stenmark H, Aasland R, Toh BH, D'Arrigo A (1996) Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. *J Biol Chem* **271**: 24048-24054.

133 Kutateladze T, Overduin M (2001) Structural mechanism of endosome docking by the FYVE domain. *Science* **291**: 1793-1796.

134 Dumas JJ, Merithew E, Sudharshan E, Rajamani D, Hayes S, Lawe D, Corvera S, Lambright DG (2001) Multivalent endosome targeting by homodimeric EEA1. *Mol Cell* **8**: 947-958.

135 Shisheva A, Sbrissa D, Ikonomov O (1999) Cloning, characterization, and expression of a novel Zn2+-binding FYVE finger-containing phosphoinositide kinase in insulin-sensitive cells. *Mol Cell Biol* **19**: 623-634.

136 Itoh T, Koshiba S, Kigawa T, Kikuchi A, Yokoyama S, Takenawa T (2001) Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. *Science* **291**: 1047-1051.

137 Mills IG, Praefcke GJ, Vallis Y, Peter BJ, Olesen LE, Gallop JL, Butler PJ, Evans PR, McMahon HT (2003) EpsinR: an AP1/clathrin interacting protein involved in vesicle trafficking. *J Cell Biol* **160**: 213-222.

138 Ford MG, Mills IG, Peter BJ, Vallis Y, Praefcke GJ, Evans PR, McMahon HT (2002) Curvature of clathrin-coated pits driven by epsin. *Nature* **419**: 361-366.

5 MANUSCRIPT I: REVIEW ARTICLE

Cell. Mol. Life Sci. 66 (2009) 876 – 883
1420-682X/09/050876-8
DOI 10.1007/s00018-009-8699-0

Cellular and Molecular Life Sciences

Review

The PAS-domain kinase PASKIN: a new sensor in energy homeostasis

P. Schläfli, E. Borter[+], P. Spielmann and R. H. Wenger[*]

Institute of Physiology and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Winterthurerstrasse 190, 8057 Zurich (Switzerland), Fax: +41 44 6356814, e-mail: roland.wenger@access.uzh.ch

Received 2 November 2008; received after revision 10 December 2008; accepted 5 January 2009
Online First 4 February 2009

Abstract. The PAS domain kinase PASKIN, also termed PAS kinase or PASK, is an evolutionarily conserved potential sensor kinase related to the heme-based oxygen sensors of nitrogen-fixing bacteria. In yeast, the two PASKIN homologs link energy flux and protein synthesis following specific stress conditions. In mammals, PASKIN may regulate glycogen synthesis and protein translation. *Paskin* knock-out mice do not show any phenotype under standard animal husbandry conditions. Interestingly, these mice seem to be protected from the symptoms of the metabolic syndrome when fed a high-fat diet. Energy turnover might be increased in specific PASKIN-deficient cell types under distinct environmental conditions. According to the current model, binding of a putative ligand to the PAS domain disinhibits the kinase domain and activates PASKIN auto- and target phosphorylation. Future research needs to be conducted to elucidate the nature of the putative ligand and the molecular mechanisms of downstream signalling by PASKIN.

Keywords. Diabetes mellitus, glucose tolerance, glycogen synthesis, insulin, metabolic syndrome, nitrogen fixation, protein translation, respiration.

Introduction

Energy homeostasis is central to life. All organisms need to adapt to nutrition availability by reducing energy expenditure when food is scarce and by storing energy when food is plentiful. Whereas many important insights have been obtained into the signalling pathways and effector mechanisms involved in metabolic energy adaptation, less is known about the actual cellular mechanisms 'sensing' primary nutritional compounds and secondary metabolic intermediates. This review focuses on PASKIN, a novel protein that contains a possibly sensory PAS domain and an effector Ser/Thr kinase domain. Recent results suggest that PASKIN serves as a sensor involved in metabolic energy homeostasis.

The PAS domain: heterodimerization interface and ligand binding site

The ~7000 PAS folds identified up to date maintain similar structures despite low sequence conservation and a large functional flexibility. The name 'PAS' is an acronym derived from the three PAS fold-containing transcription factors period (PER), aryl hydrocarbon receptor nuclear translocator (ARNT) and single-minded (SIM). PER is a component of a network of

[+] Present address: Cytos Biotechnology, 8952 Schlieren, Switzerland.
[*] Corresponding author.

transcription factors, all of them containing PAS domains, which compose the circadian clock [1]. ARNT is the common heterodimerization partner of the aryl hydrocarbon receptor (AHR), also called dioxin receptor, and the hypoxia-inducible factor (HIF) α subunits [2, 3]. SIM controls midline cell specification during neurogenesis, and *SIM* is a critical locus gene in Down syndrome [4–6].

As part of transcription factors, the PAS region usually consists of two adjacent degenerate repeats of ~130 amino acids, called PAS A and PAS B. PAS domains contain five antiparallel β-sheets flanked by α-helices (Fig. 1). In basic-helix-loop-helix (bHLH) DNA-binding proteins, the main role of the PAS region is to confer specific protein-protein interactions within the family (Fig. 1a). However, the three-dimensional structure of the PAS domain resembles a left-handed glove, and just like a baseball in a mitt, the PAS structure is also well-suited to host small-molecule ligands (Fig. 1b). Indeed, AHR was discovered based on its role in the mammalian xenobiotic response, and AHR is the only known biological receptor that binds the environmental pollutant dioxin. Neuronal NPAS2, a circadian clock component and a putative tumour suppressor, is another mammalian PAS transcription factor reported to contain a ligand. Each PAS domain of NPAS2 coordinates a heme group that specifically binds carbon monoxide [7]. Both the possible endogenous ligand of AHR and the physiological meaning of the carbon monoxide binding by NPAS2 remain under investigation. Up to date, no other mammalian transcription factors have been reported to contain ligand-binding PAS domains.

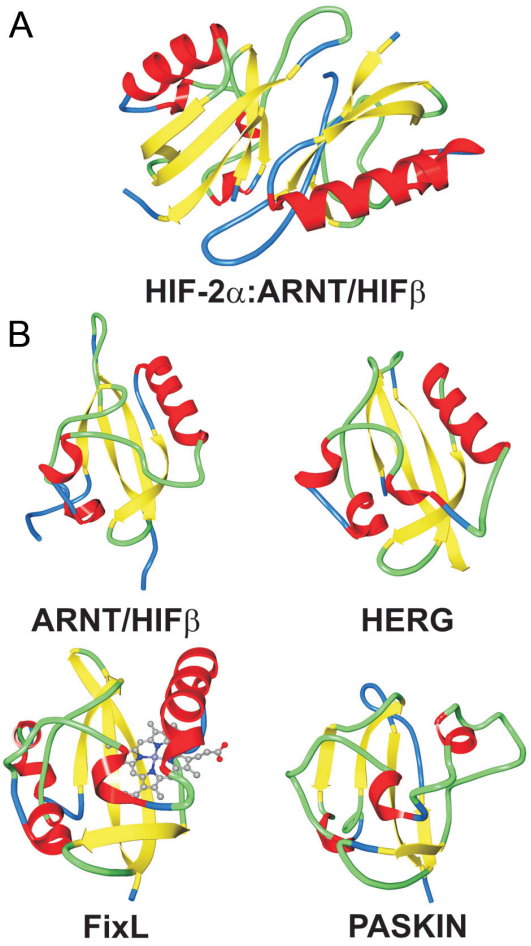


Figure 1. Structures of various PAS domains. (*A*) PAS domain heterodimerization between HIF-2α and ARNT/HIFβ. (*B*) Structural similarity between various PAS domains. Structures were prepared using Sirius1.2 software (http://sirius.sdsc.edu/) based on the following Protein Data Bank coordinates: HADDOCK structure of HIF-2α:ARNT/HIFβ heterodimer (2A24) [35]; NMR structure of ARNT/HIFβ (1X0O) [35] and PASKIN (1LL8) [20]; X-ray structure of HERG (1BYW) [12] and FixL including the heme group (1D06) [36]. ARNT, aryl hydrocarbon nuclear translocator; HERG, human *ether-à-go-go*-related gene; HIF, hypoxia-inducible factor.

The archaeal and bacterial ancestors of mammalian PAS proteins

Physiological adaptations of an organism to changing environmental conditions require molecular sensors capable of sensing and signalling specific physicochemical parameters. Especially in Bacteria and Archaea, the PAS domain is often found in environmental protein sensors involved in the perception of external signals, including light intensity, gas partial pressures, redox potentials, and certain organic ligands [8–11]. In the mammalian HERG potassium channel, the PAS domain might even sense membrane potentials [12].

Bacterial *Rhizobium* and *Bradyrhizobium* species live in root nodules of some plants, such as soybean and clover. Oxygen sensing in these species is required for regulated expression of the oxygen-sensitive enzymes involved in nitrogen fixation. The PAS domain in the histidine kinase FixL serves as a heme-bearing molecular oxygen sensor. Upon oxygen binding by the ferrous iron within the heme group, the PAS domain changes its conformation and inhibits the histidine kinase domain, regulating the activity of the FixJ transcription factor in an oxygen-dependent manner [13]. Other bacterial gas-responsive PAS proteins include the *Escherichia coli* direct oxygen sensor (*Ec*Dos) and the *Burkholderia xenovorans* regulator of CO metabolism (RcoM). Similar to eukaryotic

NPAS2, bacterial RcoM coordinates heme and binds carbon monoxide and nitric oxide [14].

PASKIN, a mammalian relative of bacterial oxygen sensors

Of physiological interest, the oxygen-regulated HIF transcription factors contain PAS domains just like the oxygen sensing FixL of nitrogen-fixing bacteria. In analogy to the binding of dioxin and carbon monoxide by the PAS domains of AHR and NPAS2, respectively, this raised the question as to whether the HIF PAS domain can sense oxygen directly. However, no evidence was found for HIF being a direct oxygen sensor. Moreover, in the meantime it became clear that oxygen sensitivity is conferred by oxygen-dependent protein hydroxylation of HIF α subunits, leading to transcriptional inactivation as well as polyubiquitination and proteasomal destruction [3, 15]. Nevertheless, this intriguing relation between the bacterial and mammalian oxygen-sensing systems raised the possibility that a mammalian homolog of the bacterial oxygen sensors might be involved in oxygen sensing in mammals. Only a single mammalian gene could be found which has a domain architecture similar to FixL, i.e. a PAS region combined with a kinase domain. We called the human and mouse genes *PASKIN* and *Paskin*, respectively [16]. Interestingly, the nucleotide sequence provides more evidence that *PASKIN* might have evolved from the FixL gene. First, the PASKIN PAS region shares a higher sequence similarity with the FixL PAS region than with any other known PAS domain. Second, a single long exon between the PAS B and serine/threonine kinase regions of *PASKIN* is weakly similar to the histidine kinase domain of FixL and only few point mutations within the introns interrupt an even longer open reading frame, suggesting that the histidine kinase domain might have been replaced by a serine/threonine kinase domain during evolution [16]. However, the presence of potential sensing and signalling domains raised the possibility that PASKIN might serve as a sensor protein.

The PAS domain of PASKIN inhibits serine/threonine kinase domain-dependent autophosphorylation

Despite the structural relation, we found heme bound to FixL but not to PASKIN (unpublished data), and there is ample evidence to rule out a role for PASKIN in direct oxygen-sensing in mammals. A first hint for another kind of functional relationship between FixL and PASKIN was provided by the group of S. L. McKnight, J. Rutter and colleagues who cloned human PASKIN and alternatively termed it PAS kinase or PASK [17]. However, since this name had been in use before for an unrelated kinase [18, 19], we herein refer to the unambiguous designation 'PASKIN' throughout this review. McKnight, Rutter and colleagues reported that PASKIN autophosphorylates the threonine residues Thr1161 and Thr1165 and that the N-terminal PAS A domain of PASKIN partially represses the C-terminal kinase activity *in trans* as well as *in cis*. Following de-repression, presumably by ligand binding to the PAS domain, autophosphorylation *in trans* results in the 'switch-on' of the kinase activity of PASKIN. Mutations of Thr1161or Thr1165 completely inactivated PASKIN kinase activity [17].

Further evidence for intrinsic kinase inhibition by the PAS domain was provided by structural studies: in contrast to other PAS proteins, the PAS A domain of PASKIN contains a short F α-helix and a long and dynamic FG loop that interacts with the kinase domain. Distinct mutations mimicking the ligand-bound state de-repressed the kinase activity without affecting the overall structure of the PAS A domain [20]. Thus, it was tempting to speculate that PASKIN binds (so far unknown) organic ligands, increases its kinase activity by autophosphorylation and signals to (so far unknown) downstream phosphoproteins.

PASKIN has also been found in a large biochemical screen for nuclear phosphoproteins in HeLa cells, confirming that PASKIN is a phosphoprotein and providing a hint for its subcellular localization [21]. In this study, PASKIN was found to be phosphorylated at Ser116. Since Ser116 is located adjacent to the PAS A domain, it potentially might regulate the PAS domain function. However, to date neither the kinase nor the functional implications of this phosphosite are known.

PSK1 and PSK2, the yeast homologs of PASKIN

Important insights into the function of PASKIN were derived from the cloning and analysis of two genes with high sequence similarity from *Saccharomyces cerevisiae*, termed PSK1 and PSK2 [22]. These budding yeast homologs of PASKIN phosphorylate three translation factors and two enzymes involved in the regulation of glycogen and trehalose synthesis, suggesting that they might coordinately control translation and sugar flux. Under specific stress conditions (a temperature-sensitive growth defect on galactose medium, called Gal^{ts} phenotype), albeit not under standard culture conditions, yeast glycogen synthase and UDP-glucose pyrophosphorylase (Ugp1) are

phosphorylated by Psk1 and Psk2, resulting in the downregulation of carbohydrate storage, whereas deficiency in Psk1 and Psk2 resulted in elevated glycogen stores. Simultaneously, Psk2-dependent phosphorylation of the yeast translation factors Caf20p (corresponding to 4E-BP in mammals), eIF1A and Sro9p leads to the downregulation of protein synthesis [22]. Further experiments revealed that the Gal^{ts} phenotype might be exclusively related to the lack of Ugp1 Ser11 phosphorylation in the PSK mutant yeast strains. Upon Ser11 phosphorylation, Ugp1 translocates to the plasma membrane where it increases cell wall glucan synthesis at the expense of glycogen storage. In the absence of PSKs, glycogen rather than glucan is produced, affecting the strength of the cell wall. This effect was fully phenocopied by a Ugp1 Ser11Ala mutation, and it could be reversed by adding the osmotic stabilizer sorbitol [23]. Two independent cell stressors have been identified to activate PSKs in yeast. Cell integrity stress (e. g. heat shock or SDS treatment) required the Wsc1 membrane stress sensor, and growth in non-glucose carbon sources (e. g. raffinose) required the AMP-dependent kinase (AMPK) homolog Snf1 [24]. While PSK2 was predominantly activated by Wsc1, PSK1 was indispensable for Snf1 function.

Because the Ser/Thr kinase domain of PASKIN is highly related to the kinase domains of AMPK family members (30 and 32 % identity over a stretch of 253 amino acids for AMPKα1 and AMPKα2, respectively), it is tempting to transfer this latter finding to the mammalian system. However, despite its relationship with bacterial FixL, PASKIN most probably does not serve as a mammalian oxygen sensor; likewise, a putative role for PASKIN as an AMPK-like energy sensor in mammalian cells needs to be approached with caution. Indeed, the PASKIN Ser/Thr kinase domain is also highly similar to the *PIM1*, *PIM2* and *PIM3* oncogenes (35, 37 and 33 % identity, respectively) and generally shares 28–37 % sequence identity with a number of other kinases of the calcium/calmodulin-dependent protein kinase family. Thus, functional predictions based only on sequence comparisons with the kinase domain are likely to be of minor relevance. Regarding the second function of PSKs in yeast, mammalian Ugp1 is not a PASKIN target, and the yeast Ugp1 Ser11 phosphorylation site is not conserved in mammalian Ugp1 [25].

Parallels between yeast and mammalian PASKIN downstream targets

The exciting findings regarding the function of the PASKIN homologs in yeast raised the question as to whether similar downstream targets phosphorylated by PASKIN might exist in mammalian cells. Indeed, the mammalian muscle glycogen synthase has subsequently been reported to be a PASKIN target [26]. Glycogen synthase is known to be regulated by a number of kinases targeting various phosphorylation sites. PASKIN inactivates glycogen synthase by phosphorylation at Ser640. The middle region of PASKIN between the PAS and kinase domains interacts with glycogen synthase. Glycogen inhibits this interaction, suggesting a glycogen-sensing function [26].

By yeast two-hybrid screening for novel protein interactors of PASKIN, we found that the eukaryotic translational elongation factor eEF1A1 is phosphorylated by PASKIN at Thr432, probably leading to increased translation elongation, at least following PASKIN overexpression [27]. Additional screenings of kinase target peptide arrays revealed novel potential targets of PASKIN that are known to be involved in the regulation of sugar flux and protein synthesis, including the ribosomal subunit S6, which is also phosphorylated by S6-kinase, a major regulator of protein translation (unpublished data). However, neither the biological stimulus nor the physiological relevance of PASKIN-dependent phosphorylation of glycogen synthase and translation factors is currently known. Thus, additional work will be required to clarify the functional relation between yeast PSK and mammalian PASKIN function.

Is the PASKIN PAS A domain binding a ligand?

The 3-dimensional structure of the PASKIN PAS A domain has been resolved, and synthetic ligands binding to this domain were identified [20]. The identified molecules are slightly reminiscent of the dioxin structure with two aromatic rings as most prominent feature. Intriguingly, ligand binding as well as ligand-mimicking mutations relieved the kinase domain from PAS domain inhibition, as is known from FixL following oxygen binding to the heme-bearing PAS domain. However, these synthetic ligands bind only with rather low affinity, and no data have been provided to date for putative cellular functions of the synthetic ligands. Thus, despite the beauty of the concept of ligand-mediated kinase de-repression, it is important to note that neither an endogenous ligand of PASKIN has been identified nor has this concept been confirmed in a cellular system, including yeast, or in animals *in vivo*.

Generation and initial characterization of *Paskin* knock-out mice

In order to better understand the physiological role of PASKIN in mammals, we targeted the mouse *Paskin* gene by homologous recombination in embryonic stem cells. Therefore, exons 10–14, forming the upstream and N-terminal parts of the kinase domain, were replaced by an IRES/*lac*Z reporter gene [28]. This loss-of-function mutation maintained normal expression of a truncated PASKIN mRNA and hooked it up to a β-galactosidase reporter. It cannot be excluded that the truncated PASKIN N-terminal region might have some biological effects, but since no suitable antibodies are available, the expression levels of this truncated protein remain unknown. However, while the PAS domain is still present, at least on the mRNA level, a potential truncated PASKIN protein will be non-functional because central regions and the kinase domain are missing.

Initial analyses revealed that PASKIN mRNA expression in wild-type mice and β-galactosidase mRNA in heterozygous animals was identical, allowing the identification of PASKIN expressing cell types by *in situ* X-gal staining for β-galactosidase activity. Surprisingly, PASKIN expression is strongly upregulated in post-meiotic germ cells during spermatogenesis [28, 29]. In fact, PASKIN mRNA in testis is at least 100-fold higher than in any other organ examined. Assuming that physiological functions and the major expression site of PASKIN will overlap, we concentrated our initial investigations on the testis. However, neither male fertility nor sperm production or motility were affected in homozygous knock-out mice. The animals do not show any gross alterations. In particular, life expectancy, food intake, energy expenditure, locomotor activity (unpublished data kindly provided by P. Wielinga, B. Alder, C. Loewenstein and T. Lutz, Zurich, Switzerland), fat distribution and liver fat content (unpublished data kindly provided by J. Hillebrand and W. Langhans, Zurich, Switzerland) and circadian activity under constant dark conditions (unpublished data kindly provided by I. Tobler, Zurich, Switzerland) are indistinguishable from control littermates. Despite glycogen synthase being a downstream target of PASKIN, periodic acid-Schiff staining did not reveal any alterations in glycogen deposits in the tissues of *Paskin* knock-out mice (unpublished data). Thus, at least under standard animal husbandry conditions *Paskin* knock-out mice do not display any developmental, morphological or behavioural phenotype.

A recent report suggested that PASKIN might be involved in respiratory regulation [30]. Female but not male *Paskin* knock-out mice showed an increased ventilatory response to acute hypoxia and failed to reach ventilatory acclimatization to chronic hypoxia. L-DOPA production as a means to determine tyrosine hydroxylase activity was decreased in catecholaminergic cells of the brainstem of male but increased in female *Paskin* knock-out mice when compared to wild-type animals. Peripheral chemoreceptors were not affected in *Paskin* knock-out mice, consistent with a lack of PASKIN expression in carotid bodies (unpublished data kindly provided by J. López-Barneo, Sevilla, Spain). Of note, body temperature, O_2 consumption and CO_2 production decreased following exposure to hypoxia, but the values were indistinguishable between *Paskin* wild-type and knock-out mice of either sex [30]. Despite its putative significance considering the bacterial ancestors of PASKIN, these studies failed to provide a mechanistic explanation for the respiratory differences. Especially the relation between tissue-specific PASKIN expression, tyrosine hydroxylase activity and the oxygenation- and sex-specific functions deserve further investigations.

A role for PASKIN in glucose-stimulated insulin production in the pancreas?

Intriguingly, it has been suggested that PASKIN kinase activity followed by mRNA and protein expression is increased in MIN6 cells and in isolated pancreatic β-cells after exposure to high glucose concentrations [31]. Increased PASKIN activity appeared to be required for glucose-dependent transcriptional induction of the pancreatic duodenal homeobox-1 (PDX-1) transcription factor, leading to transcriptional induction of preproinsulin but not glucokinase or uncoupling protein 2 gene expression. Mutations of the PDX-1 DNA binding site on both the preproinsulin promoter and the *PDX1* promoter itself inhibited glucose and PASKIN overexpression-dependent promoter activity [31]. In contrast to this *PDX1* gene-activating function of PASKIN, it has subsequently been reported by the same group that PASKIN phosphorylates PDX-1 at Thr152, which might drive PDX-1 out of the nucleus [32]. However, the authors concluded that 'decreases in PASK activity in β cells may thus contribute to some forms of type 2 diabetes, whereas activation of the enzyme may provide a new therapeutic strategy for this disease' [31].

To examine the role of PASKIN in glucose-stimulated insulin production, we used pancreatic β-cells derived from our *Paskin* wild-type and knock-out mice [33]. We found PASKIN mRNA regulation by high glucose neither in various pancreatic β-cell or testicular cell lines, nor in isolated islets or tubuli seminiferi. Increasing the ambient glucose concentration in

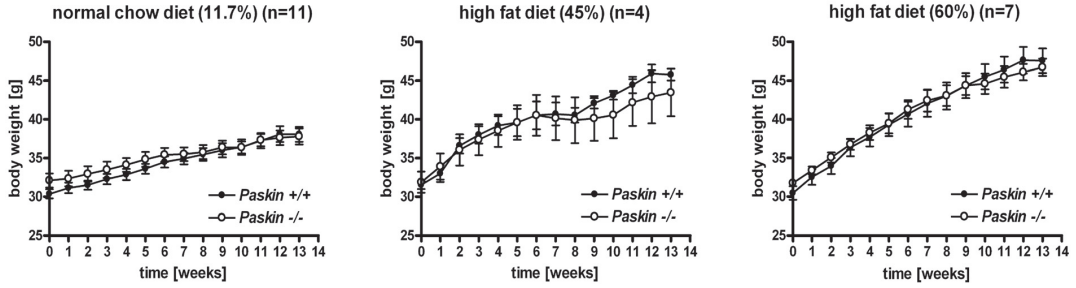


Figure 2. Body weight gain of wild-type and *Paskin* knock-out mice fed with increasingly fat-containing diets (11.7, 45 and 60 % calories by fat, respectively). Male mice received the indicated diets beginning at the age of 14 weeks. Mean values ± SEM of the indicated number of mice per group are shown.

cultured islets resulted in a similar increase in insulin mRNA and insulin secretion, whether the β-cells were derived from *Paskin* wild-type or knock-out mice. In young as well as in older *Paskin* wild-type and knock-out mice, glucose tolerance tests showed equal blood glucose clearance, whether the animals were fed a normal chow diet or fasted overnight. These experiments demonstrated normal acute and chronic insulin function in *Paskin* knock-out mice *in vivo*, leading to the conclusion that insulin expression is independent of PASKIN, at least under our laboratory conditions [33]. A lack of constitutive and glucose-induced PASKIN protein expression was confirmed in human β-cells (unpublished data kindly provided by M. Donath, Zurich, Switzerland).

Paskin knock-out mice seem to be protected from all aspects of the metabolic syndrome when fed a high-fat diet

Excitingly, when feeding mice with a high fat diet (HFD, 45 % fat by calories), J. Rutter and colleagues found that *Paskin* knock-out mice are protected from obesity, liver triglyceride accumulation, glucose intolerance and peripheral insulin resistance, the symptoms common to the metabolic syndrome [34]. These differences were accounted for by an increase in energy turnover as measured by O_2 consumption and CO_2 production. The molecular mechanisms leading to this interesting phenotype are unclear. An involvement of mitochondrial biogenesis, AMPK and mTOR activity could be excluded. As in skeletal muscle of *Paskin* knock-out mice, a partial (~50 %) PASKIN knock-down in L6 myoblasts resulted in increased glucose and fatty acid oxidation with a concomitant increase in ATP levels [34]. While these results implied a cell-autonomous effect of PASKIN on energy turnover, we did not find any differences in ATP levels in mouse embryonic fibroblasts derived from wild-type or *Paskin* knock-out mice (unpublished data), suggesting that these effects are cell type-specific. Considering the phenotype of *Paskin* knock-out mice (see above), constitutive changes in intracellular ATP production are also unlikely to occur in β-cells of the pancreas.

The lean phenotype of *Paskin* knock-out mice appeared only after feeding a HFD. Under normal chow diet (NCD), body weight, fat composition, body temperature, O_2 consumption and CO_2 production of *Paskin* knock-out mice are not distinguishable from wild-type littermates [30, 34]. After an independent repetition of the feeding studies, we could confirm that an NCD made no significant difference, whereas a HFD led to a stronger increase in body weight in wild-type mice than in *Paskin* knock-out mice (unpublished data). Rather surprisingly, when we further increased the fat content of the food (from 45 to 60 % calories by fat) there was no longer any difference in body weight increase between wild-type and *Paskin* knock-out mice (Fig. 2). Currently, we have no explanation for this unexpected finding.

It is important to mention that the same *Paskin* knock-out mouse strain was used for these experiments as for all other studies mentioned above [28]. The only notable difference was that the mice were backcrossed into C57BL/6 five times in the Hao et al. study [34], whereas we used the 10th backcross. Despite an apparently impaired glucose-stimulated insulin secretion by islets of Langerhans isolated from *Paskin* knock-out mice, glucose and insulin tolerance were not significantly different between wild-type and knock-out mice fed an NCD [34]. Moreover, in contrast to the previous report [31], PDX-1 mRNA levels were independent of PASKIN in this study [34]. While these apparently contradictory data are difficult to interpret, they confirm our previous results [33].

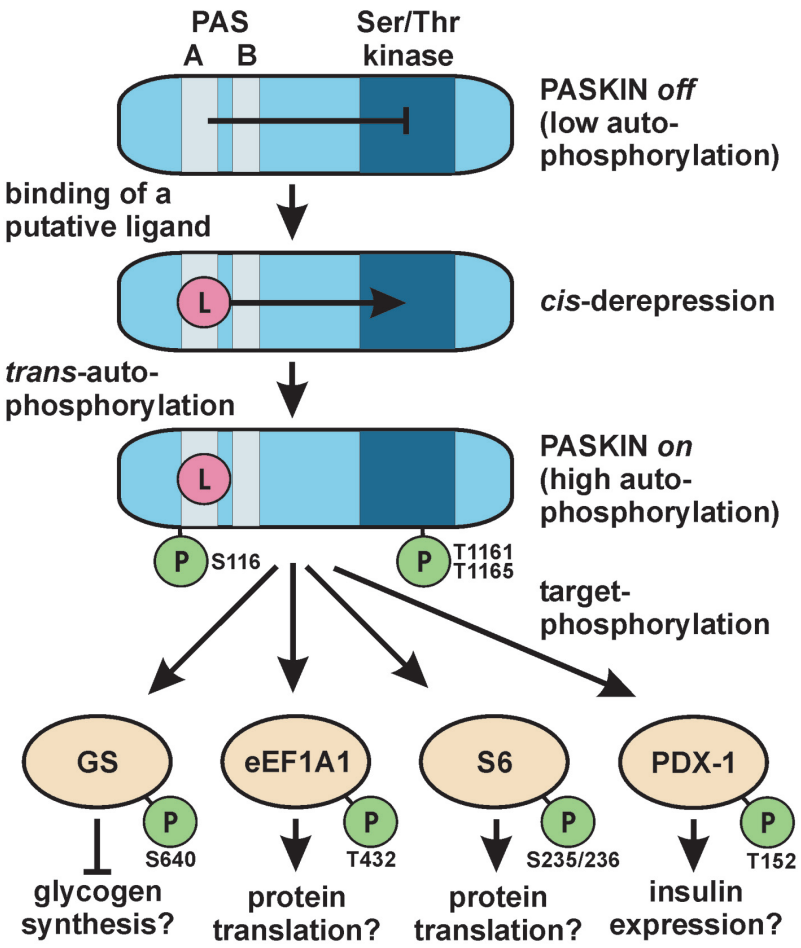


Figure 3. Model of PASKIN-dependent signalling. Upon binding of an unknown organic ligand, PASKIN autophosphorylation and target phosphorylation increase, resulting in the regulation of critical factors in energy homeostasis. GS, glycogen synthase; eEF1A1, eukaryotic translation elongation factor 1A1; PDX-1, pancreatic duodenal homeobox-1.

Conclusions

Figure 3 provides an overview of the current model of the molecular functions of PASKIN as a potential sensor protein. Much less is known about the physiological significance of PASKIN. Similar to yeast, the reports on the functions of mammalian PASKIN clearly demonstrate that PASKIN plays a role under changing environmental conditions (oxygen, glucose, nutrition) rather than under standard laboratory conditions. This might also explain the different outcomes of similar experiments in different laboratories: subtle changes in nutritional and/or other environmental parameters of the animals as well as of the cell cultures might have influenced PASKIN-dependent cellular effects. However, in contrast to the previous suggestion [31], it appears to be the decrease rather than an increase in PASKIN activity that protects from the metabolic syndrome, at least in the mouse model described in this review. PASKIN has been suggested to provide a signal for 'metabolic sufficiency' by sensing a yet unknown metabolic ligand [25, 34]. Pharmaceutical inhibition of PASKIN thus might be useful for treatment of the metabolic syndrome, especially regarding the finding that PASKIN deficiency apparently does not affect the health of knock-out mice. It will be a major challenge to identify the endogenous ligand of PASKIN. Only knowledge of the nature of this ligand will eventually clarify the role of PASKIN as an energy sensor.

Acknowledgments. Our work is supported by grants obtained from the Wolfermann-Nägeli-Stiftung, Baugarten Stiftung/Stiftung für Wissenschaftliche Forschung an der Universität Zürich and the Swiss National Science Foundation (SNF 3100AO-116047/1). The authors wish to thank C. Aerni, P. Wielinga, B. Alder, C. Loewenstein, T. Lutz, I. Tobler, J. López-Barneo, M. Donath, W. Langhans and J. Hillebrand for analysis of *Paskin* knock-out mice and sharing their unpublished data.

1 Lowrey, P. L. and Takahashi, J. S. (2000) Genetics of the mammalian circadian system: photic entrainment, circadian pacemaker mechanisms, and posttranslational regulation. Annu. Rev. Genet. 34, 533–562.
2 Mimura, J. and Fujii-Kuriyama, Y. (2003) Functional role of AhR in the expression of toxic effects by TCDD. Biochim. Biophys. Acta 1619, 263–268.
3 Wenger, R. H. (2002) Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors and O_2-regulated gene expression. FASEB J. 16, 1151–1162.
4 Chen, H., Chrast, R., Rossier, C., Gos, A., Antonarakis, S. E., Kudoh, J., Yamaki, A., Shindoh, N., Maeda, H., Minoshima, S. et al. (1995) Single-minded and Down syndrome? Nat. Genet. 10, 9–10.
5 Chrast, R., Scott, H. S., Chen, H., Kudoh, J., Rossier, C., Minoshima, S., Wang, Y., Shimizu, N. and Antonarakis, S. E. (1997) Cloning of two human homologs of the *Drosophila single-minded* gene SIM1 on chromosome 6q and SIM2 on 21q within the Down syndrome chromosomal region. Genome Res. 7, 615–624.
6 Crews, S. T. (1998) Control of cell lineage-specific development and transcription by bHLH-PAS proteins. Genes Dev. 12, 607–620.
7 Dioum, E. M., Rutter, J., Tuckerman, J. R., Gonzalez, G., Gilles-Gonzalez, M. A. and McKnight, S. L. (2002) NPAS2: a gas-responsive transcription factor. Science 298, 2385–2387.
8 Ponting, C. P. and Aravind, L. (1997) PAS: a multifunctional domain family comes to light. Curr. Biol. 7, R 674-R 677.
9 Zhulin, I. B. and Taylor, B. L. (1997) PAS domain S-boxes in archaea, bacteria and sensors for oxygen and redox. Trends Biochem. Sci. 22, 331–333.
10 Taylor, B. L. and Zhulin, I. B. (1999) PAS domains: internal sensors of oxygen, redox potential and light. Microbiol. Mol. Biol. Rev. 63, 479–506.
11 Gu, Y. Z., Hogenesch, J. B. and Bradfield, C. A. (2000) The PAS superfamily: sensors of environmental and developmental signals. Annu. Rev. Pharmacol. Toxicol. 40, 519–561.
12 Morais Cabral, J. H., Lee, A., Cohen, S. L., Chait, B. T., Li, M. and Mackinnon, R. (1998) Crystal structure and functional analysis of the HERG potassium channel N terminus: a eukaryotic PAS domain. Cell 95, 649–655.
13 Sousa, E. H., Tuckerman, J. R., Gonzalez, G. and Gilles-Gonzalez, M. A. (2007) A memory of oxygen binding explains the dose response of the heme-based sensor FixL. Biochemistry 46, 6249–6257.
14 Marvin, K. A., Kerby, R. L., Youn, H., Roberts, G. P. and Burstyn, J. N. (2008) The transcription regulator RcoM-2 from *Burkholderia xenovorans* is a cysteine-ligated hemoprotein that undergoes a redox-mediated ligand switch. Biochemistry 47, 9016–9028.
15 Schofield, C. J. and Ratcliffe, P. J. (2004) Oxygen sensing by HIF hydroxylases. Nat. Rev. Mol. Cell. Biol. 5, 343–354.
16 Hofer, T., Spielmann, P., Stengel, P., Stier, B., Katschinski, D. M., Desbaillets, I., Gassmann, M. and Wenger, R. H. (2001) Mammalian PASKIN, a PAS-serine/threonine kinase related to bacterial oxygen sensors. Biochem. Biophys. Res. Commun. 288, 757–764.
17 Rutter, J., Michnoff, C. H., Harper, S. M., Gardner, K. H. and McKnight, S. L. (2001) PAS kinase: an evolutionarily conserved PAS domain-regulated serine/threonine kinase. Proc. Natl. Acad. Sci. USA 98, 8991–8996.
18 Ushiro, H., Tsutsumi, T., Suzuki, K., Kayahara, T. and Nakano, K. (1998) Molecular cloning and characterization of a novel Ste20-related protein kinase enriched in neurons and transporting epithelia. Arch. Biochem. Biophys. 355, 233–240.
19 Miao, N., Fung, B., Sanchez, R., Lydon, J., Barker, D. and Pang, K. (2000) Isolation and expression of PASK, a serine/threonine kinase, during rat embryonic development, with special emphasis on the pancreas. J. Histochem. Cytochem. 48, 1391–1400.
20 Amezcua, C. A., Harper, S. M., Rutter, J. and Gardner, K. H. (2002) Structure and interactions of PAS kinase N-terminal PAS domain: model for intramolecular kinase regulation. Structure 10, 1349–1361.
21 Beausoleil, S. A., Jedrychowski, M., Schwartz, D., Elias, J. E., Villén, J., Li, J., Cohn, M. A., Cantley, L. C. and Gygi, S. P. (2004) Large-scale characterization of HeLa cell nuclear phosphoproteins. Proc. Natl. Acad. Sci. USA 101, 12130–12135.
22 Rutter, J., Probst, B. L. and McKnight, S. L. (2002) Coordinate regulation of sugar flux and translation by PAS kinase. Cell 111, 17–28.
23 Smith, T. L. and Rutter, J. (2007) Regulation of glucose partitioning by PAS kinase and Ugp1 phosphorylation. Mol. Cell 26, 491–499.
24 Grose, J. H., Smith, T. L., Sabic, H. and Rutter, J. (2007) Yeast PAS kinase coordinates glucose partitioning in response to metabolic and cell integrity signaling. EMBO J. 26, 4824–4830.
25 Hao, H. X. and Rutter, J. (2008) The role of PAS kinase in regulating energy metabolism. IUBMB Life 60, 204–209.
26 Wilson, W. A., Skurat, A. V., Probst, B., de Paoli-Roach, A., Roach, P. J. and Rutter, J. (2005) Control of mammalian glycogen synthase by PAS kinase. Proc. Natl. Acad. Sci. USA 102, 16596–16601.
27 Eckhardt, K., Tröger, J., Reissmann, J., Katschinski, D. M., Wagner, K. F., Stengel, P., Paasch, U., Hunziker, P., Borter, E., Barth, S. et al. (2007) Male germ cell expression of the PAS domain kinase PASKIN and its novel target eukaryotic translation elongation factor eEF1A1. Cell. Physiol. Biochem. 20, 227–240.
28 Katschinski, D. M., Marti, H. H., Wagner, K. F., Shibata, J., Eckhardt, K., Martin, F., Depping, R., Paasch, U., Gassmann, M., Ledermann, B. et al. (2003) Targeted disruption of the mouse PAS domain serine/threonine kinase PASKIN. Mol. Cell. Biol. 23, 6780–6789.
29 Wenger, R. H. and Katschinski, D. M. (2005) The hypoxic testis and post-meiotic expression of PAS domain proteins. Semin. Cell. Dev. Biol. 16, 547–553.
30 Soliz, J., Soulage, C., Borter, E., van Patot, M. T. and Gassmann, M. (2008) Ventilatory responses to acute and chronic hypoxia are altered in female but not male *Paskin*-deficient mice. Am. J. Physiol. 295, R649–658.
31 da Silva Xavier, G., Rutter, J. and Rutter, G. A. (2004) Involvement of Per-Arnt-Sim (PAS) kinase in the stimulation of preproinsulin and pancreatic duodenum homeobox 1 gene expression by glucose. Proc. Natl. Acad. Sci. USA 101, 8319–8324.
32 An, R., da Silva Xavier, G., Hao, H. X., Semplici, F., Rutter, J. and Rutter, G. A. (2006) Regulation by Per-Arnt-Sim (PAS) kinase of pancreatic duodenal homeobox-1 nuclear import in pancreatic beta-cells. Biochem. Soc. Trans. 34, 791–793.
33 Borter, E., Niessen, M., Zuellig, R., Spinas, G. A., Spielmann, P., Camenisch, G. and Wenger, R. H. (2007) Glucose-stimulated insulin production in mice deficient for the PAS kinase PASKIN. Diabetes 56, 113–117.
34 Hao, H. X., Cardon, C. M., Swiatek, W., Cooksey, R. C., Smith, T. L., Wilde, J., Boudina, S., Abel, E. D., McClain, D. A. and Rutter, J. (2007) PAS kinase is required for normal cellular energy balance. Proc. Natl. Acad. Sci. USA 104, 15466–15471.
35 Card, P. B., Erbel, P. J. and Gardner, K. H. (2005) Structural basis of ARNT PAS-B dimerization: use of a common β-sheet interface for hetero- and homodimerization. J. Mol. Biol. 353, 664–677.
36 Miyatake, H., Mukai, M., Park, S. Y., Adachi, S., Tamura, K., Nakamura, H., Nakamura, K., Tsuchiya, T., Iizuka, T. and Shiro, Y. (2000) Sensory mechanism of oxygen sensor FixL from *Rhizobium meliloti:* crystallographic, mutagenesis and resonance Raman spectroscopic studies. J. Mol. Biol. 301, 415–431.

6 MANUSCRIPT II: ORIGINAL ARTICLE

Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2007;20:227-240

Accepted: February 02, 2007

Male Germ Cell Expression of the PAS Domain Kinase PASKIN and its Novel Target Eukaryotic Translation Elongation Factor eEF1A1

Katrin Eckhardt,[1,§,*] Juliane Tröger,[1,§] Jana Reissmann,[2] Dörthe M. Katschinski,[2,3] Klaus F. Wagner,[4,5] Petra Stengel,[4] Uwe Paasch,[6] Peter Hunziker,[7] Emanuela Borter,[1] Sandra Barth,[1] Philipp Schläfli,[1] Patrick Spielmann,[1] Daniel P. Stiehl,[1] Gieri Camenisch[1] and Roland H. Wenger[1]

[1]Institute of Physiology and Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich, [2]Cell Physiology Group, Medical Faculty, Martin-Luther-University Halle, [3]Heart and Circulatory Physiology, Georg August University Göttingen, [4]Institute of Physiology and [5]Clinic of Anaesthesiology, University of Lübeck, [6]European Training Center of Andrology, University of Leipzig, and [7]Functional Genomics Center Zürich, University of Zürich, [§]The first two authors contributed equally to this publication, *Present address: Institute of Cell Biology, ETH Zürich

Key Words
Energy homeostasis • Glycogen synthesis • Nitrogen fixation • Protein phosphorylation • Protein translation • Testis

Abstract
PASKIN links energy flux and protein synthesis in yeast, regulates glycogen synthesis in mammals, and has been implicated in glucose-stimulated insulin production in pancreatic β-cells. Using newly generated monoclonal antibodies, PASKIN was localized in the nuclei of human testis germ cells and in the midpiece of human sperm tails. A speckle-like nuclear pattern was observed for endogenous PASKIN in HeLa cells in addition to its cytoplasmic localization. By yeast two-hybrid screening, we identified the multifunctional eukaryotic translation elongation factor eEF1A1 as a novel interaction partner of PASKIN. This interaction was mapped to the PAS A and kinase domains of PASKIN and to the C-terminus of eEF1A1 using mammalian two-hybrid and GST pull-down assays. Kinase assays, mass spectrometry and site-directed mutagenesis revealed PASKIN auto-phosphorylation as well as eEF1A1 target phosphorylation mainly but not exclusively at Thr432. Wild-type but not kinase-inactive PASKIN increased the *in vitro* translation of a reporter cRNA. Whereas eEF1A1 did not localize to the nucleus, it co-localizes with PASKIN to the cytoplasm of HeLa cells. The two proteins also showed a remarkably similar localization in the midpiece of the sperm tail. These data suggest regulation of eEF1A1 by PASKIN-dependent phosphorylation in somatic as well as in sperm cells.

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/07/0204-0227$23.50/0

Accessible online at:
www.karger.com/cpb

R. H. Wenger
Institute of Physiology, University of Zürich
Winterthurerstrasse 190, CH-8057 Zürich (Switzerland)
Tel. +41 44 6355065, Fax +41 44 6356814
E-Mail roland.wenger@access.uzh.ch

Introduction

Physiological adaptations of an organism to changing environmental conditions require molecular sensors capable of sensing and signalling specific physico-chemical parameters. The PAS (Per-Arnt-Sim) domain is a widespread protein fold of environmental protein sensors involved in the perception of light intensity, oxygen partial pressure, redox potentials, voltage and certain ligands [1]. In mammals, the PAS domain is mainly found as a heterodimerization interface of transcription factors involved in the molecular circadian clock, dioxin toxicity and oxygen sensing [2-4].

We and others previously identified a novel mammalian PAS protein, termed PASKIN [5] or PAS kinase [6]. The domain architecture of PASKIN resembles that of the oxygen sensor protein FixL from nitrogen-fixing *Rhizobium* species. PASKIN contains two PAS domains (PAS A and PAS B) and a serine/threonine kinase domain related to AMP kinases which might be regulated in *cis* by binding of so far unknown ligands to the PAS domain [7]. Following de-repression, autophosphorylation in *trans* results in the "switch-on" of the kinase domain of PASKIN [6]. The budding yeast PASKIN homologs PSK1 and PSK2 phosphorylate three translation factors and two enzymes involved in the regulation of glycogen and trehalose synthesis, thereby coordinately controlling translation and sugar flux [8]. Under stress conditions (nutrient restriction combined with high temperature), PASKIN kinase activity results in increased protein synthesis and decreased carbohydrate storage in yeast. PASKIN-dependent phosphorylation also inhibits the activity of the mammalian glycogen synthase [9]. In addition, a recent report suggested that PASKIN expression as well as kinase activity is increased in isolated pancreatic -cells following stimulation with high glucose levels [10]. Increased PASKIN activity appeared to be required for glucose-dependent transcriptional induction of preproinsulin gene expression, which might be related to PASKIN-dependent regulation of the nuclear import of pancreatic duodenal homeobox-1 transcription factor [11].

We recently generated PASKIN null mice by targeted replacement of the kinase domain of the mouse *Paskin* gene by a *lacZ-neo* fusion construct in embryonic stem cells [12, 13]. Surprisingly, PASKIN expression is strongly upregulated in post-meiotic germ cells during spermatogenesis as revealed by *in situ* hybridization, galactosidase staining and mRNA blotting. In fact, PASKIN mRNA levels in testis are several magnitudes higher than in all other organs tested. However, at least under laboratory conditions, fertility as well as sperm production and sperm motility were not affected in PASKIN knock-out mice. No other organs, including pancreas, stained positive for galactosidase, and we could not detect any PASKIN-dependent insulin regulation [14].

To obtain more insights into PASKIN function, we generated specific monoclonal antibodies derived against PASKIN and screened a HeLa cDNA expression library in a yeast two-hybrid system to identify novel PASKIN interaction partners in mammals. Here we show that the eukaryotic translational elongation factor eEF1A1 interacts with PASKIN. eEF1A1 is a GTP-binding protein catalyzing the binding of charged aminoacyl-tRNA to the A-site of the ribosome [15-17]. eEF1A1 is of particular interest because it is related to the yeast translation initiation factor eIF1A that mediates the transfer of Met-tRNA to the 40S subunit of the ribosome, and which was shown to be regulated by the yeast PASKIN homologs PSK1 and PSK2 [8].

Materials and Methods

Plasmids

If not indicated otherwise, cloning work was carried out using Gateway technology (Invitrogen, Basel, Switzerland). pcDNA3hPASK [6], containing wild-type or mutant human PASKIN cDNA (kindly provided by J. Rutter, Salt Lake City, UT, USA), was digested with *Nco*I (all restriction enzymes were purchased from MBI Fermentas, Labforce, Nunningen, Switzerland), blunted with Klenow polymerase and subcloned into *Sal*I-*Eco*RV-blunted pENTR4 (Invitrogen) to obtain pENTR4hPASK. The PAS domain plasmid pENTR4PAS was obtained by *Bam*HI digestion and re-ligation of pENTR4hPASK. The kinase (KIN) domain plasmid pENTR4KIN was obtained by subcloning the *Ecl*136II fragment of pcDNA3hPASK into the *Xmn*I-*Eco*RV sites of pENTR4. Other fragments of PASKIN were amplified by PCR using Pfu polymerase (MBI Fermentas), digested with *Nco*I and *Xho*I and subcloned into the same sites of pENTR4. The following primers (synthesized by Microsynth, Balgach, Switzerland) were used: PAS A (5'-cat gcc atg gta agt gtg tcc tgc tgc tcc ct-3' and 5'-cta gct cga gtt act gcc gca tcc tct tca tcc-3'); PAS B (5'-cat gcc atg gct tgg gtg ttc tgc acc atc-3' and 5'-cta gct cga gtt agg cca ggt ctg gga gct gta-3'); N-terminal (5'-cag gac gcc cgc cat aaa ct-3' and 5'-cta gct cga gtt atg agg acc acc ctg-3'); centerpiece (5'-cat gcc atg gag atc cga aag ctg atg gaa-3' and 5'-cta gct cga gtt act cag cag cgg tag agt gg-3'). pENTR4PASA1/2 was obtained by digesting pENTR4PASKIN with *Bam*HI and *Hin*dIII, Klenow fill-in and re-ligation. The plasmid pCMV6-XL5-eEF1A1, containing the full-length human eEF1A1 cDNA, was purchased from OriGene (Rockville, MD, USA). eEF1A1 was amplified by PCR (full-length: 5'-cat gcc atg gga aag gaa

aag act cat atc-3' and 5'-cta gct cga gcc gtt ctt cca cca ctg att-3'; 1-241: 5'-aag cag aag gcc atc ctg ac-3' and 5'-cta gct cga gtt atg gac gag ttg gtg gta gga-3'; 247-462: 5'-cat gcc atg gtg cgc ctg cct ctc cag gat-3' and 5'-gat atc tcg agc cgt tct tcc ac-3') and subcloned into pENTR4 as above. The T432A mutation was introduced by Pfu polymerase-based site-directed mutagenesis using the primer 5'-cgt gat atg aga cag gct gtt gcg gtg ggt g-3', followed by *Dpn*I digestion of the parental template. The inserts of all ENTRy vectors were verified by DNA sequencing (Microsynth). To generate expression vectors for fusion proteins, ENTRy vectors were recombined *in vitro* with DESTination vectors using LR Clonase recombination enzyme mix (Invitrogen). pDEST15 and pDEST17 were used to generate bacterial or rabbit reticulocyte lysate expression vectors for GST- and His_6-fusion proteins, respectively. pDEST10 and pDEST20 were used to generate expression vectors for His_6- and GST-fusion proteins, respectively, in the baculovirus/Sf9 insect cell system (Invitrogen). pcDNA3.1/nV5-DEST was used to express N-terminal V5-tagged proteins in mammalian cells or in rabbit reticulocyte lysates.

Generation of monoclonal antibodies

GST-PAS fusion protein expression was induced in *E. coli* BL21-AI by 0.2% arabinose for 4 hours and affinity purified with glutathione sepharose (Amersham Biosciences, Dübendorf, Switzerland). Two mice were immunized with this antigen using standard procedures. Hybridoma cell lines were established and culture supernatant tested against the antigen, or GST alone, by ELISA and immunoblotting. Antibodies from positive hybridoma supernatants were purified using protein A agarose (EconoPac protein A cartridge, BioRad, Reinach, Switzerland) liquid chromatography. Antibody isotyping was performed using ISOstrip mouse strips (Roche Diagnostics, Mannheim, Germany).

Immunoblotting

Combined cytoplasmic and nuclear extracts of cultured cells were prepared using 0.4 M NaCl and 0.1% NP-40 in extraction buffer as described previously [18]. Nuclear extracts were prepared from isolated nuclei using 0.4 M NaCl. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard. Immunoblotting and chemiluminescence detection was performed as described previously [19]. The following antibodies were used: mouse monoclonal antibody (mAb) Gal4 and mAb VP16 (Santa Cruz Biotechnology, Heidelberg, Germany); mAb eEF1A1 (Stressgen, Biomol, Hamburg, Germany); mAb V5 tag (Invitrogen); mAb His_6 tag (Abcam, Cambridge, UK); rabbit polyclonal β-actin (Sigma); and secondary polyclonal goat anti-mouse or anti-rabbit antibodies coupled to horseradish peroxidase (Pierce, Perbio, Lausanne, Switzerland).

Immunohistochemistry

Paraffin-embedded human testis samples (tumor orchidectomy) were sectioned (2 µm) and antigen retrieved by boiling the sections in 0.1 M citrate buffer for 90 minutes. Peroxidase activity was blocked by incubating with 3% H_2O_2 and unspecific binding sites were blocked with protein-block (Dako, Hamburg, Germany). The slides were incubated with PASKIN mAb2 or mAb6, or eEF1A1 mAb, diluted 1:50 and 1:100, respectively, in 50 mM Tris-HCl pH 7.6, 0.5 M NaCl, 0.1% Tween 20, 10% FCS for 15 minutes. Following washing, primary antibodies were detected with the CSA enhancing system (Dako) and DAB (for PASKIN) or FastRed (for eEF1A1) substrates. PASKIN production in SF9 was detected as described above except that the Envision system (Dako) with FastRed as chromogen was employed. All slides were counterstained with hemalaun.

Immunofluorescence

Collection of human ejaculated spermatozoa was approved by the ethics committee of the University of Leipzig (approval number 067-2005). Spermatozoa were washed in PBS, streaked onto microscope slides, incubated for 5 minutes in demembranization buffer (2% Triton-X100, 5 mM DTT, 50 mM Tris-HCl pH 9.0) and fixed with 3% paraformaldehyde. The non-specific binding sites were blocked with 3% BSA in PBS for 30 minutes. Spermatozoa were incubated for 1 hour with PASKIN mAb2 or mAb6 diluted 1:10 in 3% BSA in PBS followed by a TexasRed-coupled secondary anti-mouse antibody (Dako) or an AlexaFluor 488-coupled secondary anti-mouse antibody (Invitrogen) diluted 1:100 with 3% BSA in PBS. HeLa cells were grown on cover slips and treated accordingly. Finally, nuclei were stained with Hoechst33258 dye for 5 minutes. After extensive washings with PBS, the slides were mounted and analyzed by fluorescence microscopy (Axioplan 2000, Carl Zeiss Vision, Mannheim, Germany) or confocal microscopy (LSM510, Carl Zeiss Vision).

Cell culture, transient transfection and co-immunoprecipitation

Human Hep3B hepatoma, HeLa cervical carcinoma and NCCIT male germ tumor (kind gift of S. Schweyer, Göttingen, Germany) cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (Sigma) as described previously [20]. Transient transfection was performed with the polyethylenimine method as described before [19]. Transfection efficiency usually reached 60-80% as determined by green fluorescent protein expression (data not shown). For co-immunoprecipitation, pre-cleared cell extracts were incubated with PASKIN mAb6 covalently coupled to sepharose A beads [21]. The beads were washed with 0.5% NP-40, 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM EDTA and bound proteins were analyzed by immunoblotting.

Yeast two-hybrid

A HeLa cell-derived cDNA library was screened using the PAS domain of human PASKIN as bait according to the instructions provided by the manufacturer (Clontech). Therefore, the full-length human PASKIN cDNA clone pDKFZp434O1522 [5] was digested with *Nco*I and *Sma*I and the cDNA fragment inserted into the *Nco*I-*Sma*I sites of the pAS2 vector (Clontech). Subsequently, the C-terminal part was deleted by *Bam*HI digestion and re-ligation to obtain pAS2PAS.

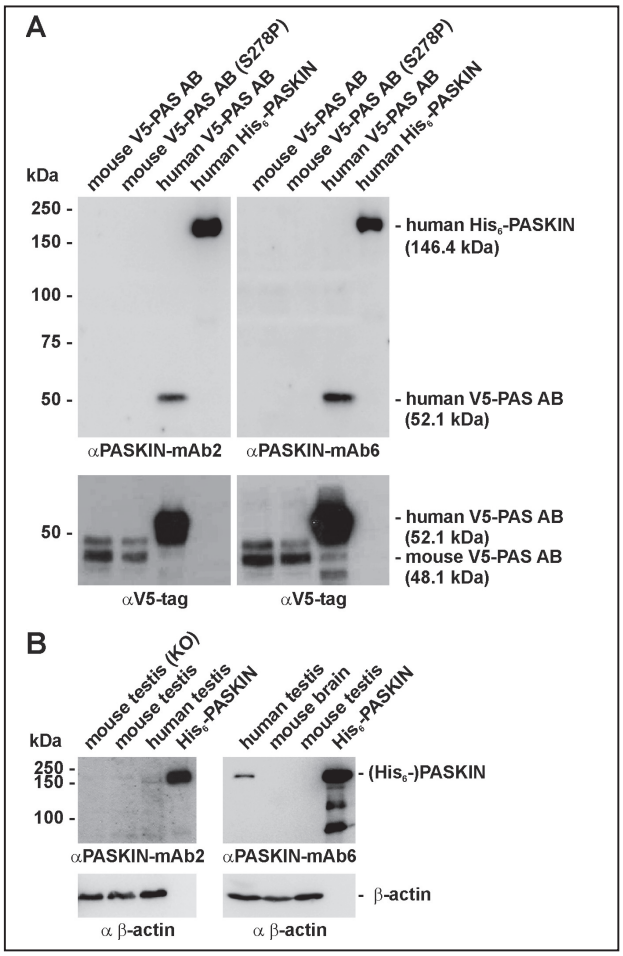


Fig. 1. Generation of two monoclonal antibodies (mAbs) specific for human PASKIN. (A) Mouse monoclonal PASKIN antibodies mAb2 and mAb6 were purified and used for immunoblot detection of human and two different clones of the mouse V5-tagged PAS AB fragments produced by IVTT and full-length human His_6-PASKIN expressed in Sf9 cells. The same blot was subsequently incubated with an anti-V5 tag antibody as control. (B) PASKIN wild-type or knock-out (KO) mouse and human tissue extracts were probed with mAb6 by immunoblotting. An extract from Sf9 cells expressing His_6-PASKIN was included as positive control. The same blot was subsequently incubated with an anti-β-actin antibody as control. Note that the PASKIN mAbs react with human but not mouse PASKIN.

Mammalian two-hybrid

The mammalian Matchmaker vectors pM and pVP16 (Clontech) were converted to DESTination vectors by ligation of the Gateway vector conversion cassette reading frame B (Invitrogen) into the *Eco*RI sites (blunted with Klenow polymerase) of pM and pVP16 to obtain pMGAL4BDattb and pVP16ADattb, respectively. Mammalian expression vectors for Gal4 DNA-binding domain (BD) or VP16 activation domain (AD) fusion proteins were obtained after *in vitro* recombination with the corresponding ENTRy vectors. The pG5FL firefly luciferase reporter gene vector was obtained by replacing the *Sal*I-*Xba*I SEAP cDNA fragment from pG5SEAP (Clontech) with the *Sal*I-*Xba*I luciferase cDNA amplified by PCR (primers 5'-gat ccg tcg act cta gca tgg aag acg cca aaa aca-3' and 5'-gct cta gaa tta cac ggc gat ctt tcc-3') from pGL3Basic (Promega, Madison, WI). Hep3B cells were transiently co-transfected with the BD and AD fusion protein vectors, the firefly luciferase reporter vector and the pRL-SV40 renilla luciferase reporter vector (Promega) to control for differences in transfection efficiency. Luciferase reporter gene activity was determined as described before [22]. Ratios between firefly and renilla luciferase activities were normalized to negative control co-transfections with the pM-53 and pVP16-CP vectors (Clontech) which were arbitrarily defined as 1.

In vitro transcription/translation (IVTT) and GST pull-down

IVTT reactions were carried out as described by the manufacturer (Promega) using recombined DESTination vectors in the presence of ^{35}S-Met (Hartmann Analytic, Braunschweig,

Germany). For translation assays, separate IVTT reactions (1 µl), containing the pDEST17 expression vector alone, galactosidase expressing *lacZ* expression vector, wild-type or mutant PASKIN, were mixed with fresh reticulocyte lysate (12.5 µl) and 12.5 ng renilla luciferase cRNA (Promega). Luciferase activity was determined as above. GST-tagged proteins or GST alone were expressed in *E. coli* BL21-AI by induction with 0.2% arabinose for 4 hours and affinity purified using glutathione sepharose columns (GSTrap FF, Amersham Biosciences) by liquid chromatography (BioLogic DuoFlow, BioRad). Pull-down experiments were performed by mixing either purified proteins or 20 µl wheat germ IVTT reactions with 10 µg purified GST-tagged proteins or GST alone bound to glutathione sepharose beads. After 30 minutes incubation at room temperature in bead binding buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.01% NP40), beads were washed 3 times with washing buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.5% NP40), boiled in sample buffer (40 mM Tris-HCl pH 6.8, 1% SDS, 50 mM β-mercaptoethanol) for 5 minutes and the proteins separated by SDS-PAGE. Gels were stained with coomassie blue, dried and radioactively labelled proteins detected by phosphorimaging (Molecular Imager FX, BioRad).

Kinase assays and mass spectrometry

His_6-PASKIN was purified from baculovirus-infected Sf9 insect cells using Ni-NTA agarose (Qiagen, Basel, Switzerland). His_6-PASKIN was incubated with 2 µg bacterially expressed and purified GST-tagged eEF1A1 (full-length or fragments) or GST alone in 25 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT for 20 minutes in the presence of 5 µCi (γ^{33}P)ATP (Hartmann Analytic). Proteins were separated by SDS-PAGE and analyzed by phosphorimaging of the dried gels. To determine the phosphorylation site, GST-eEF1A1 was incubated with ATP and PASKIN, separated by SDS-PAGE and excised from the gel. One third of the gel band was cut into small pieces and washed twice with 100 µl 100 mM NH_4HCO_3, 50% acetonitrile and once with 50 µl acetonitrile. GST-eEF1A1 was digested in-gel with 30 µl trypsin solution (modified trypsin, Promega, 10 fg/µl in 50 mM Tris-HCl pH 8.2, 2 mM $CaCl_2$) at 37°C over night. The supernatant was removed and the gel pieces were extracted twice with 100 µl 0.1% TFA, 50% acetonitrile. All three supernatants were combined in autosampler vials, dried, and dissolved in 10 µl 0.1% formic acid for LC/ESI/MS/MS analysis run in the neutral loss mode for phosphopetides (Q-TOF Ultima API, Waters/Micromass, Manchester, UK; equipped with a capLC, Waters).

Results

Generation and characterization of monoclonal antibodies against PASKIN

In order to determine the physiological sites of expression *in vivo* as well as the subcellular localization of mammalian PASKIN, monoclonal antibodies (mAbs) derived against PASKIN were generated. Therefore, a GST-PAS AB fusion protein was expressed in *E. coli*, purified and used for the immunization of mice. mABs were purified from hybridoma cell lines which reacted with GST-PAS AB but not GST alone. Two PASKIN IgG_{2a} mAbs, mAb2 and mAb6, recognized *in vitro* transcribed and translated (IVTT) human V5-PAS AB as well as His_6-PASKIN by immunoblotting (Fig. 1A). However, none of the mAbs reacted with mouse PAS AB fragments, while V5-tag antibodies readily detected the recombinant proteins (Fig. 1A), demonstrating that these mAbs do not cross-react with mouse PASKIN. In human testis extracts both mAbs detected a single band that co-migrated with recombinant His_6-PASKIN isolated from baculovirus-infected Sf9 cells (predicted MW: 146.4 kDa), suggesting that endogenous human PASKIN (predicted MW: 143.7 kDa) is either not or only slightly post-translationally modified in the testis (shown for PASKIN mAb6 in Fig. 1B). No corresponding band could be observed in mouse organs, including testis and brain (Fig. 1B).

PASKIN protein localization in germ cells of the testis

We previously reported that the mouse *Paskin* gene is expressed in the testis at least 100-fold stronger than in any other organ tested [12]. As shown above, also immunoblotting revealed PASKIN protein only in the testis. Thus, PASKIN mAb2 and mAb6 were used to analyze the PASKIN protein localization in human testis by immunohistochemistry. Signals for PASKIN were obtained in the outermost layer of cells in human seminiferous tubules, corresponding to the self-replicating spermatogonia, and in spermatocytes and round spermatids (Fig. 2A and C). At higher magnifications it became apparent that apart from some cytoplasmic staining PASKIN localized to the nuclei of spermatogonia and spermatocytes with a pattern that might match the nucleoli, at least in spermatogonia (Fig. 2B and D). In ejaculated human sperm cells, PASKIN localized mainly to the midpiece of the tail and was absent in the nucleus as determined by immunofluorescence (Fig. 2E and F).

Cytoplasmic and nuclear localization of PASKIN in cultured cells

Ectopically overexpressed V5-PASKIN had originally been reported to localize to the cytosol of transfected HEK293 cells [6]. Likewise, we detected exogenously overexpressed human His_6-PASKIN exclusively in the cytoplasm of baculovirus-infected Sf9 insect cells by immunohistochemistry using PASKIN mAb6 (Fig. 3A).

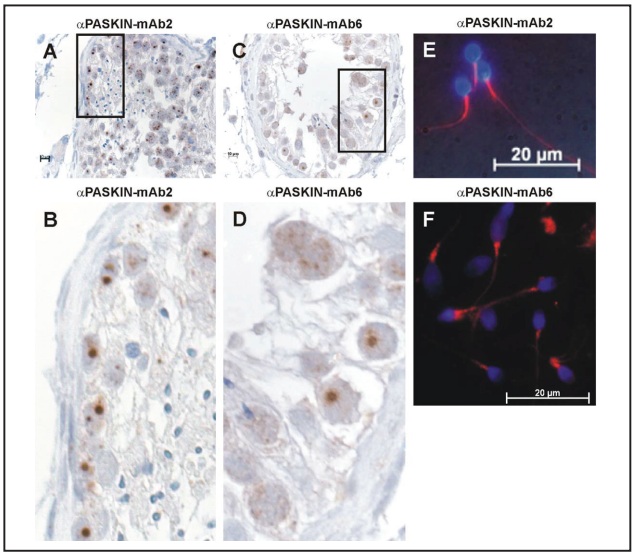


Fig. 2. Subcellular localization of PASKIN in human testis and spermatozoa. Indirect immunohistochemistry of human testis (A to D) and indirect immunofluoresence of ejaculated human sperm cells (E, F). The primary αPASKIN antibodies are indicated; the secondary antibodies were coupled to HRP (A to D) or Texas red (E, F).

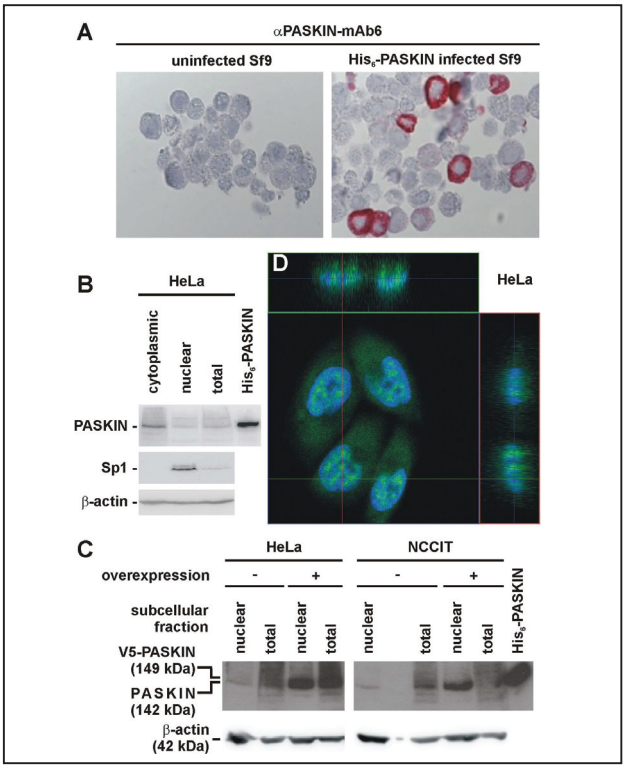


Fig. 3. PASKIN localizes to the cytoplasm as well as to nuclear speckle-like structures in cultured HeLa cells. (A) Uninfected Sf9 cells, or Sf9 cells infected with human His_6-PASKIN-baculovirus for 50 hours, were fixed, pelleted, embedded in paraffin, sectioned and analyzed by immunhistochemistry using PASKIN mAb6. (B) Immunoblotting of cytoplasmic, nuclear or total cell extracts derived from HeLa cells using PASKIN mAb6. Subsequent incubation with antibodies derived against the transcription factor Sp1 confirmed the identity of the extracts. (C) Immunoblotting of nuclear or total cell extracts derived from untransfected HeLa and NCCIT cells, or cells transfected with a V5-PASKIN expression vector, using PASKIN mAb6. Subsequent detection of actin served as control for equal loading and blotting (B, C). (D) Confocal indirect immunofluorescence microscopy of untransfected HeLa cells using αPASKIN mAb6. Nuclei were counterstained with Hoechst33258. The optical XZ and YZ planes are indicated on the sides of the XY picture to demonstrate the intranuclear localization of PASKIN.

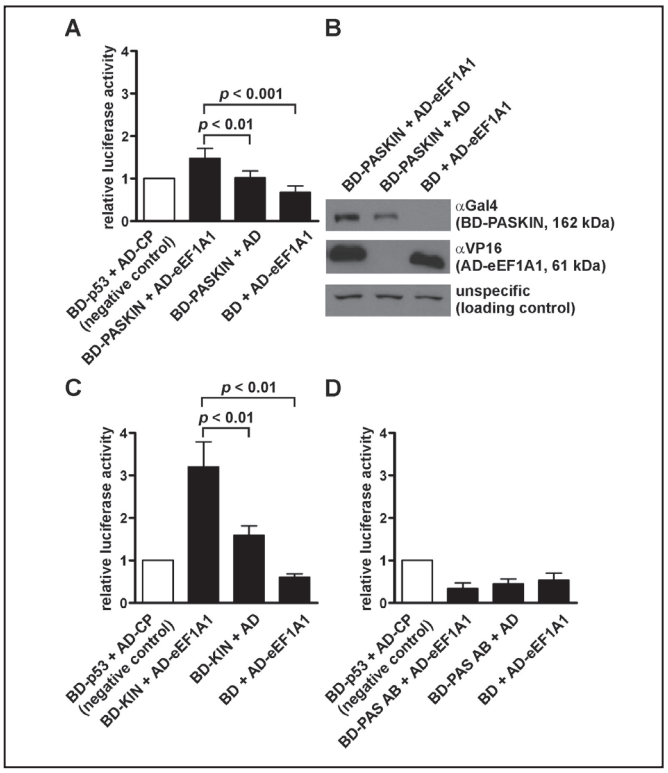


Fig. 4. PASKIN:eEF1A1 interaction in Hep3B cells. Fusion proteins between VP16-AD and full-length PASKIN (A), or the KIN domain (C), or the PAS AB domain (D), together with Gal4-BD and eEF1A1 were co-transfected into Hep3B cells together with a firefly luciferase reporter gene, containing Gal4 DNA-binding sites, and a renilla luciferase control vector. Firefly luciferase reporter gene activity was determined 24 hours later, corrected for the control renilla luciferase activity and normalized to the values obtained with a negative control transfection of a non-interacting fusion protein pair. (B), Immunoblotting of transfected Hep3B cells to confirm expression of the fusion proteins. Anti-Gal4 mAb detected the BD and anti-VP16 mAb detected the AD. An unspecific band reacting with the anti-Gal4 mAb served to control for equal loading. Mean values ± SEM are shown of 7 (A, C) or 3 (D) independent experiments performed in duplicates. *P* values were obtained by paired t tests and considered significant if $p < 0.05$.

However, by biochemical separation and immunoblotting, endogenous PASKIN had also been partially detected in the nuclear fraction of HeLa cells [6]. Moreover, a recent high-throughput screen identified PASKIN in nuclear extracts derived from HeLa cells [23]. Because PASKIN showed an unexpected nuclear pattern in human testis, we analyzed subcellular PASKIN localization in HeLa human cervical carcinoma cells as well as in NCCIT human male germ tumor cells. As shown by immunoblotting with PASKIN mAb6, endogenous PASKIN could be detected in nuclear fractions derived from these cell lines in addition to the cytoplasmic fractions (Fig. 3B). Over-expression markedly increased the portion of PASKIN in the nuclear fractions (Fig. 3C). Confocal microscopy of HeLa cells using PASKIN mAb6 revealed a cytoplasmic as well as a nuclear PASKIN localization, not overlapping with areas that stained weakly with Hoechst33258 DNA stain (Fig. 3D). At present it is unclear whether the nuclear speckle-like PASKIN pattern in HeLa cells corresponds to the nucleolar-like pattern in spermatogonia. The cytoplasmic PASKIN staining overlapped neither with markers for the golgi apparatus (wheat germ agglutinin and concanavalin A) nor mitochondria (mitotracker) (data not shown). Taken together, these data show that PASKIN can localize to the nucleus as well as to the cytoplasm.

The eukaryotic translation elongation factor eEF1A1 interacts with PASKIN

In order to better understand the function of mammalian PASKIN, a yeast two-hybrid screen for novel proteins interacting with PASKIN was performed. eEF1A1 was identified as a prey when a region spanning the PAS A and B domains was used as bait. In order to confirm this interaction, mammalian two-hybrid experiments were performed in Hep3B human hepatoma cells co-transfected with Gal4 BD-PASKIN and VP16 AD-eEF1A1 fusion constructs. The activity of a co-transfected luciferase reporter gene construct containing five Gal4 DNA-binding sites is greatly enhanced when

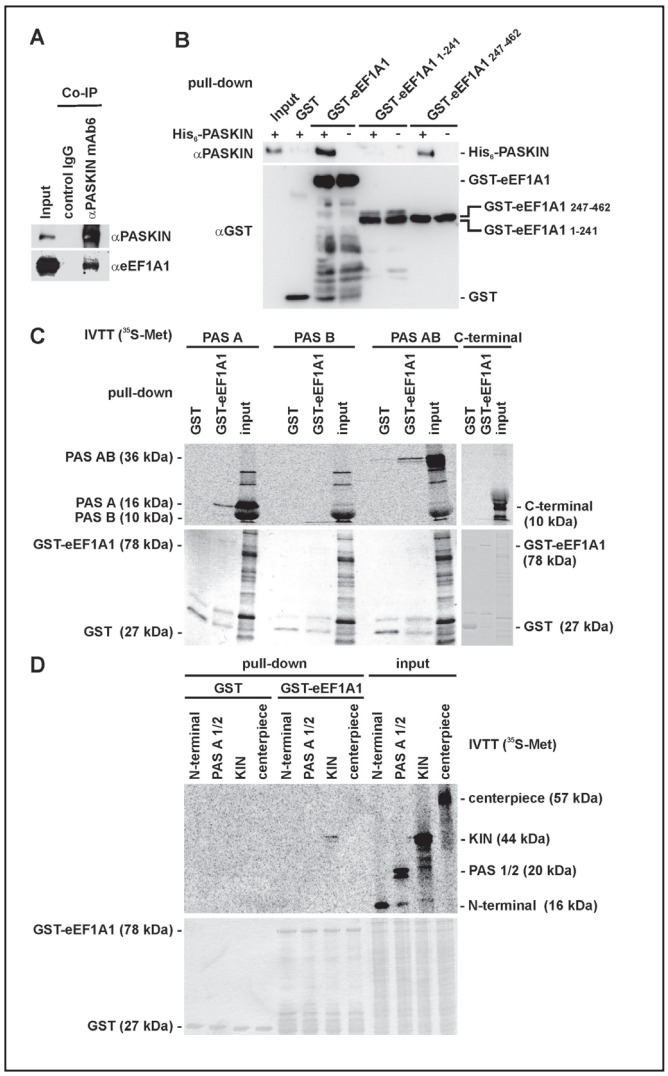


Fig. 5. PASKIN:eEF1A1 protein-protein interaction. (A) Co-immunoprecipitation of endogenous eEF1A1 with c-myc-tagged PASKIN expressed in HeLa cells. PASKIN mAb6 but not control antibodies co-precipitated eEF1A1 as shown by immunoblotting. (B) Purified recombinant proteins derived from *E. coli* (GST alone or GST-tagged eEF1A1, full-length or the indicated fragments) or Sf9 insect cells (His-PASKIN) were mixed, incubated and analyzed by GST pull-down with glutathione-sepharose followed by immunoblotting using PASKIN and GST mAbs. (C, D) Protein:protein interaction between radioactively labelled fragments of PASKIN produced by IVTT and purified recombinant GST-eEF1A1 fusion protein, or GST alone, were analyzed by GST pull-down with glutathione-sepharose followed by SDS-PAGE and phosphorimaging (upper panels in C and D). „Input" reflects fractions of the IVTT reactions before GST pull-down. The coomassie-stained gels are shown in the lower panels of (C) and (D) to demonstrate equal pull-down efficiency. Note that the full-length GST-eEF1A1 fusion protein is barely visible in (C).

the AD comes to lie in the vicinity of the BD. As shown in Fig. 4A, luciferase expression was significantly higher when the BD-PASKIN and AD-eEF1A1 fusion constructs were co-transfected compared with co-transfection of either the BD or the AD alone. Expression of the exogenous fusion proteins was confirmed by immunoblotting with anti-Gal4 or anti-VP16 antibodies (Fig. 4B). As shown in Fig. 4C, the KIN domain of PASKIN conferred even a stronger induction of luciferase activity than the full-length protein. Intriguingly, the two PAS domains did not stimulate luciferase expression but rather inhibited it when compared with the non-interacting negative control transfections (Fig. 4D). Thus, these results confirmed the interaction between the full-length PASKIN and eEF1A1 proteins, but showed that the PAS domains of PASKIN were inhibitory in the

mammalian eEF1A1 interaction assay whereas they were activatory in the yeast eEF1A1 interaction assay.

Mapping of the PASKIN domains interacting with eEF1A1

The interaction between endogenous eEF1A1 and PASKIN was further confirmed by co-immunoprecipitation. Because only very low amounts of endogenous PASKIN are expressed in HeLa cells (see Fig. 3B), c-myc-tagged PASKIN was transiently overexpressed and immunoprecipitated with PASKIN mAb6. As shown in Fig. 5A, mAb6 but not an isotype-matched control IgG co-precipitated endogenous eEF1A1.

The PASKIN:eEF1A1 interaction was then characterized by GST pull-down experiments *in vitro*. Therefore, purified full-length His_6-PASKIN was co-precipitated together with full-length, N-terminal or the C-terminal GST-tagged eEF1A1 using glutathione sepharose (Fig. 5B). Immunoblotting with PASKIN mAb6 detected His_6-PASKIN bound to full-length and C-terminal GST-eEF1A1 but not to N-terminal GST-eEF1A1 or GST alone (Fig. 5B, top), while GST antibodies detected all proteins on the same blot (Fig. 5B, bottom).

To map the PASKIN site(s) interacting with eEF1A1, fragments of PASKIN were transcribed and translated in wheat germ extracts and radioactively labelled by incorporation of ^{35}S-Met. These fragments were tested for interaction with purified GST-eEF1A1 fusion proteins, or GST alone, by precipitation with glutathione sepharose. In this assay, the PAS A, PAS AB (Fig. 5C) as well as the KIN (Fig. 5D) domains interacted with eEF1A1, whereas the N-terminal half of PAS A, PAS B, the N- and C-termini and the piece between the PAS and KIN domains (centerpiece) did not interact with eEF1A1.

In conclusion, both the C-terminal part of the PAS A domain as well as the KIN domain independently interact with the C-terminal part of eEF1A1, providing an explanation why eEF1A1 could interact with the PAS domain of PASKIN in yeast and with the KIN domain of PASKIN in Hep3B cells.

eEF1A1 is phosphorylated by PASKIN at Thr432

We next determined whether eEF1A1 can be phosphorylated by PASKIN. Therefore, His_6-PASKIN was purified from baculovirus-infected Sf9 insect cells. As expected from published results [6], His_6-PASKIN auto-phosphorylated in the presence of ^{33}P-ATP, demonstrating that it was functionally active (Fig. 6A). PASKIN

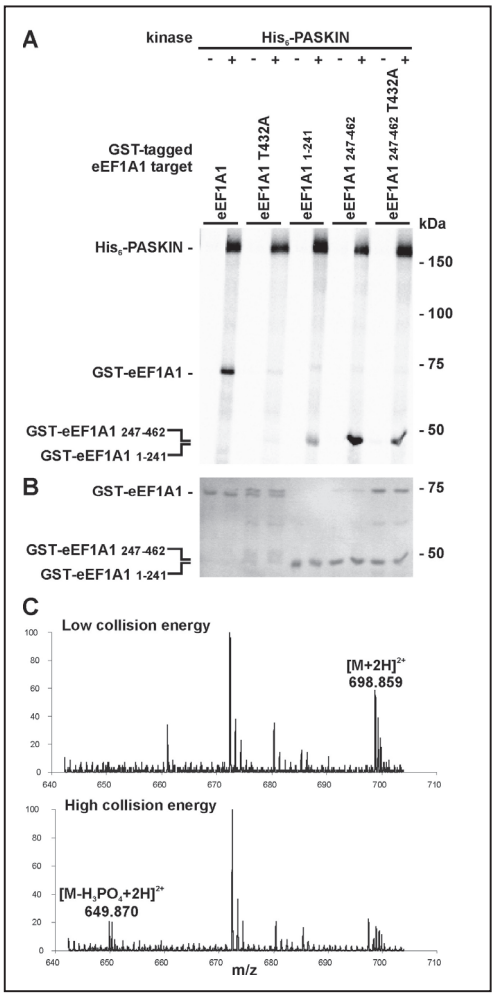


Fig. 6. PASKIN auto-phosphorylation and eEF1A1 target-phosphorylation. (A) Purified recombinant GST-eEF1A1 was *in vitro* phosphorylated by His-PASKIN, separated by SDS-PAGE and detected by phosphorimaging. T432A mutants and fragments of eEF1A1 were included as indicated. (B) Corresponding coomassie-stained gel to indicate equimolar loading of phosphorylation target proteins. (C) Neutral loss measurement by LC/ESI/MS/MS of phospho-eEF1A1 following in-gel digestion. The mass difference of 48.989 between the measurements of the peptide DMRQTVAVGVIK at low collision energy (upper trace) and at high collision energy (lower trace) shows the presence of a phosphorylation site in this peptide. Thr432 was identified as the main PASKIN-dependent phosphorylation site in eEF1A1.

also phosphorylated full-length GST-eEF1A1 and GST-eEF1A1$_{247-462}$, whereas GST-eEF1A1$_{1-241}$ was phosphorylated to a clearly lower extent (Fig. 6A) and GST alone was not phosphorylated at all (data not shown). As shown by coomassie staining, all recombinant GST-tagged proteins were present in approx. equimolar concentrations (Fig. 6B), whereas the concentration of the kinase was too low to be detected by this technique.

To determine the phosphoacceptor site of eEF1A1, full-length GST-eEF1A1 was phosphorylated by His$_6$-PASKIN, separated by SDS-PAGE and in-gel digested with trypsin. Separation of the fragments by liquid chromatography followed by electrospray ionization-tandem mass spectrometry in the neutral loss mode for phosphopetides identified the C-terminal peptide DMRQTVAVGVIK with Thr432 as the only phosphoacceptor site in this analysis (Fig. 6C).

To confirm the role of Thr432 it was mutated to alanine by site-directed mutagenesis. As shown in Fig. 6A, phosphorylation of full-length GST-eEF1A1T432A by His$_6$-PASKIN was almost completely abolished. However, while GST-eEF1A1$_{247-462}$T432A also showed impaired phosphorylation by His$_6$-PASKIN, some residual phosphorylation of this eEF1A1 fragment was still detectable, suggesting that T432A is the major but not the only phosphoacceptor site on eEF1A1.

PASKIN increases translation efficiency in a cell-free in vitro assay

PASKIN-dependent phosphorylation of eEF1A1 suggests that PASKIN might influence translation efficiency. To test for this hypothesis, wild-type and kinase-inactive mutants of PASKIN were synthesized by IVTT in rabbit reticulocyte lysates (Fig. 7A). PASKIN was then tested for its effects on translation of a renilla luciferase cRNA in fresh rabbit reticulocyte lysates containing endogenous eEF1A1 (see Fig. 8A). While the empty IVTT expression vector, galactosidase, and kinase-inactive PASKIN T1161A and/or T1165A mutants [6] did not significantly alter protein synthesis, wild-type PASKIN increased renilla luciferase protein synthesis by 80% (Fig. 7B).

Co-localization of PASKIN and eEF1A1 in the midpiece of the sperm tail

Functional interaction between PASKIN and eEF1A1 requires a cellular co-localization. Thus, we next analyzed the subcellular localization of eEF1A1. Therefore, HeLa cells were analyzed by immunofluorescence using an eEF1A1 mAb. Immunoblotting of IVTT reticulocyte extracts, containing or not containing an eEF1A1 expression vector, and of HeLa total cell extracts demonstrated the presence of eEF1A1 in HeLa cells and confirmed the specificity of the antibody (Fig. 8A). Indirect immunofluorescence with this antibody followed by an Alexa488-coupled secondary antibody localized eEF1A1 exclusively to the cytoplasm (Fig. 8B). A similar cytoplasmic localization was observed in germ cells of the human testis by immunohistochemistry (Fig. 8C). Interestingly, by indirect immunofluorescence using a Texas red-coupled secondary antibody eEF1A1 was also de-

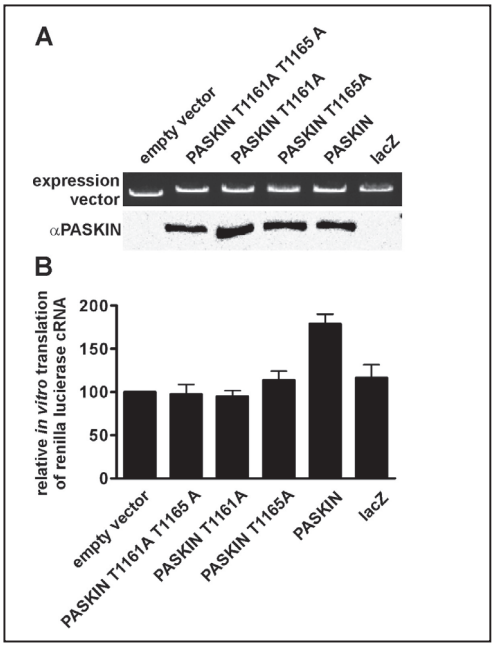


Fig. 7. PASKIN increases translation in a cell-free translation assay. (A) Wild-type PASKIN or the indicated kinase-inactive PASKIN mutants were produced by IVTT. Linearized expression vectors (agarose gel electrophoresis and ethidium bromide staining; top panel) were used to produce similar amounts of PASKIN protein (immunoblotting using PASKIN mAb6; bottom panel). (B) Translation was assayed by adding fractions of these IVTT reactions to reticulocyte lysates containing renilla luciferase cRNA. Luciferase activities were normalized to the empty vector control and are shown as mean values ± SEM of n=3 independent experiments. IVTT reactions using a *lacZ* vector expressing galactosidase served as negative controls.

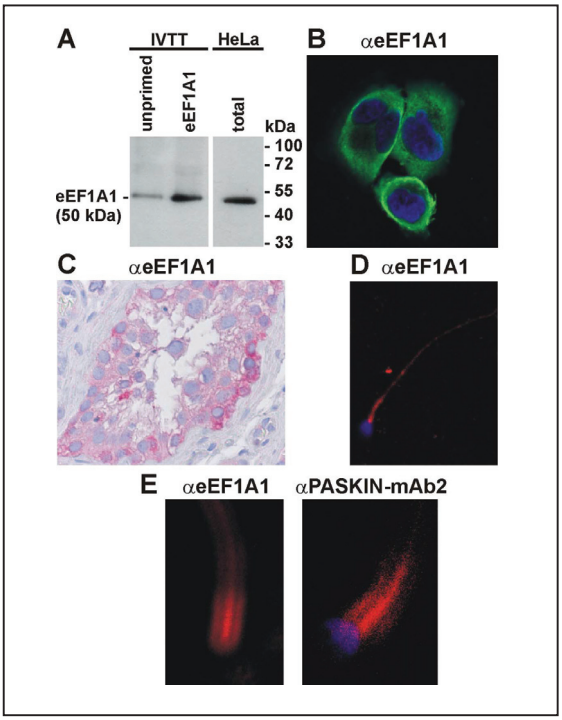


Fig. 8. eEF1A1 and PASKIN co-localization in HeLa and sperm cells. (A) Immunoblot analysis of eEF1A1 in unprimed reticulocyte lysates, or following IVTT of an eEF1A1 expression vector, and in untransfected HeLa cells, demonstrating the specificity of the eEF1A1 mAb. (B) Confocal indirect immunofluorescence microscopy of untransfected HeLa cells using eEF1A1 primary and Alexa488-coupled secondary antibodies. (C) Immunohistochemistry of human testis using eEF1A1 mAb (red). Note that both in HeLa (B) as well as in testis (C), eEF1A1 localizes to the cytoplasm, suggesting that this is the main site of interaction with PASKIN in these cells. (D) Indirect eEF1A1 immunofluorescence microscopy of human ejaculated sperm using eEF1A1 primary and Texas red-coupled secondary antibodies. (E) Confocal indirect immunofluorescence microscopy of the midpiece of the human sperm tail using eEF1A1 or PASKIN mAb6 primary and Texas red-coupled secondary antibodies. Nuclei were counterstained with DAPI (B) or Hoechst33258 (D, E).

tected in the tail of ejaculated human sperm (Fig. 8D). Confocal immunofluorescence microscopy revealed a very similar pattern of eEF1A1 and PASKIN, suggesting co-localization of the two proteins in the midpiece of the sperm tail (Fig. 8E).

Discussion

We previously identified PASKIN as a gene that is ubiquitously expressed at low abundance in most mouse organs analyzed. In contrast, much higher mRNA levels were found in the testis [5, 12]. Presumably, these low levels in somatic tissues can further be induced by changing environmental conditions, as suggested by the recent demonstration that high glucose induces PASKIN in pancreatic cells [10]. Of note, in a large-scale characterization of nuclear phosphoproteins, a PASKIN-derived phosphopeptide (Ser116) was identified in HeLa cells, suggesting a potentially regulable function for PASKIN [23]. Thus, HeLa appeared to be a valuable tool to identify PASKIN downstream targets in non-germline cells, and we identified the translation elongation factor eEF1A1 as a novel PASKIN interaction partner by yeast two-hybrid screening.

We further demonstrated that the C-terminal part of eEF1A1 interacts with both the PAS A and KIN domains of PASKIN as schematically summarized in Fig. 9. Human eEF1A1 is phosphorylated by PASKIN at Thr432, suggesting that PASKIN regulates eEF1A1 function. Several other kinases are known to phosphorylate eEF1A1, including PKC, Rho-associated kinase, and S6 kinase [24-26]. Interestingly, PKC has been shown previously to phosphorylate the corresponding Thr431 PASKIN target site in mouse eEF1A1 [24], and we found PKC-dependent human eEF1A1 phosphorylation at Thr432 (data not shown). Up to date the functional consequences of this phosphorylation remain unknown. Insulin stimulation of protein synthesis involves S6 kinase-dependent eEF1A1 phosphorylation [26], and here we could demonstrate that PASKIN increases protein translation in a cell-free translation assay. Thus, PASKIN-dependent phosphorylation of eEF1A1 might link energy metabolism with protein translation in mammalian cells

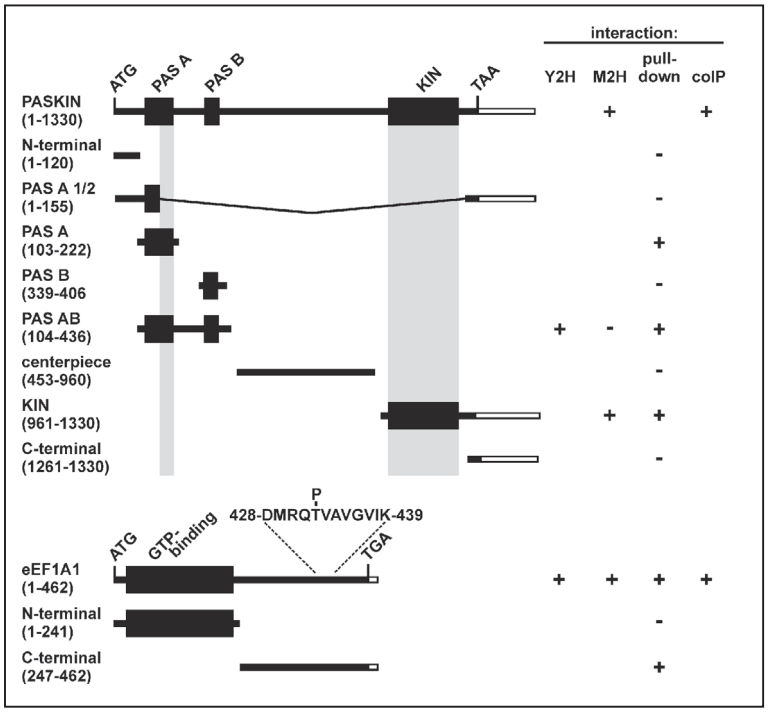


Fig. 9. Scheme of the PASKIN:eEF1A1 interaction. Results from Figs. 4 and 5 are summarized on the right part of the picture. The eEF1A1 phosphopeptide identified following PASKIN-dependent phosphorylation is indicated. Y2H, yeast two-hybrid; M2H, mammalian two-hybrid; coIP, co-immunoprecipitation.

as it has been demonstrated previously for yeast [8]. However, the major site of PASKIN expression *in vivo* is the testis. Because no corresponding cell culture model is available, at present we can only speculate on a role for PASKIN in protein translation in male germ cells.

In addition to protein translation, PASKIN-dependent eEF1A1 phosphorylation might also have completely different consequences. In fact, eEF1A1 displays a multitude of functions unrelated to protein synthesis, including cytoskeletal organization, signal transduction, RNA synthesis, proteasomal degradation of damaged proteins, apoptosis and activation of the heat-shock transcription factor; eEF1A1 hence is involved in major diseases such as diabetes and cancer [15, 17, 27]. We thus reasoned that the identification of the subcellular compartments which show co-localization of eEF1A1 and PASKIN might give further hints to the physiological functions of PASKIN-dependent eEF1A1 phosphorylation. In baculovirus-infected insect cells, we found PASKIN exclusively in the cytoplasm, consistent with a previous report on transiently transfected HEK293 cells [6]. Cytoplasmic localization of endogenous PASKIN was also found in HeLa cells, suggesting a role for the PASKIN:eEF1A1 interaction in this compartment. However, in order to study the major physiological site of PASKIN expression, the testis, it was necessary to generate specific PASKIN antibodies. Unexpectedly, endogenous PASKIN showed an apparently nucleolar pattern in spermatogonia and a more speckled nuclear pattern in spermatocytes and in HeLa cells in addition to the cytoplasmic localization. A previously not recognized phosphorylation site of PASKIN (Ser116) has been identified in nuclear extracts derived from HeLa cells [23]. It is currently unknown whether phosphorylation of PASKIN Ser116 affects PASKIN subcellular localization, PASKIN kinase activity, or both.

Although eEF1A1 has also been reported to localize to the nucleus under certain circumstances [15], we detected endogenous eEF1A1 exclusively in the cytoplasm of HeLa cells and germ cells of human testis. However, there was a complete, and thus far not recognized, overlap in PASKIN and eEF1A1 expression and locali-

zation in mature human spermatozoa. Because these cells ceased protein translation, a translation-unrelated function of PASKIN-dependent eEF1A1 phosphorylation appears to be likely. The midpiece of the sperm tail is a highly organized structure comprising cytoskeletal components, the mitochondria, and most of the remaining cytoplasmic liquid which contains several testis-specific isoforms of glycolytic enzymes as well as of the transcription factor HIF-1, another PAS domain protein [13, 28, 29]. Thus, this site is involved in the response to external stimuli by regulation of energy flux, heat-stress response and apoptosis, features that would be consistent with the known functions of PASKIN and eEF1A1. Future experiments will be required to identify the external stimuli affecting these features.

Acknowledgements

The authors wish to thank R. Fischer for the generation of monoclonal antibodies; J. Rutter, J. Nesper, S. Hägele and S. Schweyer for the generous gifts of plasmids and cells; U. Ziegler and J. Rohrer for helpful advice and discussions; and A.-K. Hellberg, B. Stier and C. Franke for excellent technical assistance. This work was supported by grants from the BMBF (NBL3 to D.M.K.), the DFG (Ka 1269/7-1 to D.M.K.), the Forschungsschwerpunkt Reproduktionsmedizin of the University of Lübeck (to K.F.W.), the Hartmann-Müller Stiftung (to R.H.W.) and the SNF (3100A0-104219 to R.H.W. and G.C.).

References

1 Ponting CP, Aravind L: PAS: a multifunctional domain family comes to light. Current Biol 1997;7:R674-R677.
2 Lowrey PL, Takahashi JS: Genetics of the mammalian circadian system: Photic entrainment, circadian pacemaker mechanisms, and posttranslational regulation. Annu Rev Genet 2000;34:533-562.
3 Mimura J, Fujii-Kuriyama Y: Functional role of AhR in the expression of toxic effects by TCDD. Biochim Biophys Acta 2003;1619:263-268.
4 Wenger RH, Stiehl DP, Camenisch G: Integration of oxygen signaling at the consensus HRE. Sci STKE 2005;2005:re12.
5 Hofer T, Spielmann P, Stengel P, Stier B, Katschinski DM, Desbaillets I, Gassmann M, Wenger RH: Mammalian PASKIN, a PAS-serine/threonine kinase related to bacterial oxygen sensors. Biochem Biophys Res Commun 2001;288:757-764.
6 Rutter J, Michnoff CH, Harper SM, Gardner KH, McKnight SL: PAS kinase: An evolutionarily conserved PAS domain-regulated serine/threonine kinase. Proc Natl Acad Sci U S A 2001;98:8991-8996.
7 Amezcua CA, Harper SM, Rutter J, Gardner KH: Structure and interactions of PAS kinase N-terminal PAS domain: model for intramolecular kinase regulation. Structure 2002;10:1349-1361.
8 Rutter J, Probst BL, McKnight SL: Coordinate regulation of sugar flux and translation by PAS kinase. Cell 2002;111:17-28.
9 Wilson WA, Skurat AV, Probst B, de Paoli-Roach A, Roach PJ, Rutter J: Control of mammalian glycogen synthase by PAS kinase. Proc Natl Acad Sci U S A 2005;102:16596-16601.
10 da Silva Xavier G, Rutter J, Rutter GA: Involvement of Per-Arnt-Sim (PAS) kinase in the stimulation of preproinsulin and pancreatic duodenum homeobox 1 gene expression by glucose. Proc Natl Acad Sci U S A 2004;101:8319-8324.
11 An R, da Silva Xavier G, Hao HX, Semplici F, Rutter J, Rutter GA: Regulation by Per-Arnt-Sim (PAS) kinase of pancreatic duodenal homeobox-1 nuclear import in pancreatic beta-cells. Biochem Soc Trans 2006;34:791-793.
12 Katschinski DM, Marti HH, Wagner KF, Shibata J, Eckhardt K, Martin F, Depping R, Paasch U, Gassmann M, Ledermann B, Desbaillets I, Wenger RH: Targeted disruption of the mouse PAS domain serine/threonine kinase PASKIN. Mol Cell Biol 2003;23:6780-6789.
13 Wenger RH, Katschinski DM: The hypoxic testis and post-meiotic expression of PAS domain proteins. Semin Cell Dev Biol 2005;16:547-553.
14 Borter E, Niessen M, Zuellig R, Spinas GA, Spielmann P, Camenisch G, Wenger RH: Glucose-stimulated insulin production in mice deficient for the PAS kinase PASKIN. Diabetes 2007;56:113-117.
15 Ejiri S: Moonlighting functions of polypeptide elongation factor 1: from actin bundling to zinc finger protein R1-associated nuclear localization. Biosci Biotechnol Biochem 2002;66:1-21.
16 Browne GJ, Proud CG: Regulation of peptide-chain elongation in mammalian cells. Eur J Biochem 2002;269:5360-5368.
17 Lamberti A, Caraglia M, Longo O, Marra M, Abbruzzese A, Arcari P: The translation elongation factor 1A in tumorigenesis, signal transduction and apoptosis: review article. Amino Acids 2004;26:443-448.

18 Katschinski DM, Le L, Schindler SG, Thomas T, Voss AK, Wenger RH: Interaction of the PAS B domain with HSP90 accelerates hypoxia-inducible factor-1α stabilization. Cell Physiol Biochem 2004;14:351-360.

19 Stiehl DP, Wirthner R, Köditz J, Spielmann P, Camenisch G, Wenger RH: Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J Biol Chem 2006;281:23482-23491.

20 Katschinski DM, Le L, Heinrich D, Wagner KF, Hofer T, Schindler SG, Wenger RH: Heat induction of the unphosphorylated form of hypoxia-inducible factor-1α is dependent on heat shock protein-90 activity. J Biol Chem 2002;277:9262-9267.

21 Harlow E, Lane D: Using antibodies. A laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1999.

22 Martin F, Linden T, Katschinski DM, Oehme F, Flamme I, Mukhopadhyay CK, Eckhardt K, Tröger J, Barth S, Camenisch G, Wenger RH: Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. Blood 2005;105:4613-4619.

23 Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villén J, Li J, Cohn MA, Cantley LC, Gygi SP: Large-scale characterization of HeLa cell nuclear phosphoproteins. Proc Natl Acad Sci U S A 2004;101:12130-12135.

24 Kielbassa K, Müller HJ, Meyer HE, Marks F, Gschwendt M: Protein kinase Cδ-specific phosphorylation of the elongation factor eEF- and an eEF-1 peptide at threonine 431. J Biol Chem 1995;270:6156-6162.

25 Izawa T, Fukata Y, Kimura T, Iwamatsu A, Dohi K, Kaibuchi K: Elongation factor-1 is a novel substrate of Rho-associated kinase. Biochem Biophys Res Commun 2000;278:72-78.

26 Chang YW, Traugh JA: Phosphorylation of elongation factor 1 and ribosomal protein S6 by multipotential S6 kinase and insulin stimulation of translational elongation. J Biol Chem 1997;272:28252-28257.

27 Shamovsky I, Ivannikov M, Kandel ES, Gershon D, Nudler E: RNA-mediated response to heat shock in mammalian cells. Nature 2006;440:556-560.

28 Marti HH, Katschinski DM, Wagner KF, Schäffer L, Stier B, Wenger RH: Isoform-specific expression of hypoxia-inducible factor-1α during the late stages of mouse spermiogenesis. Mol Endocrinol 2002;16:234-243.

29 Depping R, Hägele S, Wagner KF, Wiesner RJ, Camenisch G, Wenger RH, Katschinski DM: A dominant-negative isoform of hypoxia-inducible factor-1α specifically expressed in human testis. Biol Reprod 2004;71:331-339.

7 MANUSCRIPT III (SUBMITTED)

Substrate preference and phosphatidylinositol monophosphate inhibition of the catalytic domain of the PAS kinase PASKIN

Philipp Schläfli[1], Juliane Tröger[1,2], Katrin Eckhardt[3], Emanuela Borter[4], Patrick Spielmann, and Roland H. Wenger

Institute of Physiology and Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich, CH-8057 Zürich, Switzerland

[1]Both authors contributed equally to this work.

Present addresses
[2]Division of Digestive and Liver Diseases, Department of Medicine, Columbia University, New York 10032, USA
[3]Institute of Cell Biology, ETH Zürich, CH-8093 Zürich, Switzerland
[4]Biogen-Dompé, CH-6300 Zug, Switzerland.

Correspondence
R. H. Wenger, Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. Tel.: +41 (0)44 6355065; Fax: +41 (0)44 6356814; E-mail: roland.wenger@access.uzh.ch; URL: http://www.physiol.uzh.ch

Running title
Targets and stimulation of PASKIN

Abbreviations
DAG, diacylglycerol; DOG, dioctanoyl glycerol; eEF1A1, eukaryotic elongation factor 1A1; mTOR, mammalian target of rapamycin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PH, pleckstrin homology; PKA/PKC, protein kinase A/C; PLC/PLD, phospholipase C/D; PS, phosphatidylserine; PSK, protein Ser/Thr kinase; PtdIns, phosphatidylinositol; S6K, S6 kinase; Snf1, sucrose nonfermenting 1; TOP, terminal oligopyrimidine; Ugp1, UDP-glucose pyrophosphorylase 1.

Keywords
Metabolism, phospholipid; protein translation; sensory kinase; ribosomal protein S6

SUMMARY

The PAS domain serine/threonine kinase PASKIN, or PAS kinase, links energy flux and protein synthesis in yeast, regulates glycogen synthesis and protein translation in mammals, and might be involved in insulin regulation in the pancreas. According to the current model, binding of a putative ligand to the PAS domain disinhibits the kinase domain, leading to PASKIN autophosphorylation and increased kinase activity. Up to date, only synthetic but no endogenous PASKIN ligands have been reported. Here, we identified a number of novel PASKIN kinase targets, including ribosomal protein S6. Together with our previous identification of eukaryotic translation elongation factor eEF1A1, this suggests a role for PASKIN in the regulation of mammalian protein translation. While searching for endogenous PASKIN ligands, we found that various phospholipids can bind PASKIN and stimulate its autophosphorylation. Interestingly, strongest binding and autophosphorylation was achieved with monophosphorylated phosphatidylinositols. However, stimulated PASKIN autophosphorylation did not correlate with ribosomal protein S6 and eEF1A1 target phosphorylation. Whereas autophosphorylation was enhanced by monophosphorylated phosphatidylinositols, di- and tri-phosphorylated phosphatidylinositols inhibited autophosphorylation. In contrast, target phosphorylation was always inhibited, with highest efficiency of di- and tri-phosphorylated phosphatidylinositols. Since phosphatidylinositol monophosphates were found to interact with the kinase rather than with the PAS domain, these data suggest a multi-ligand regulation of PASKIN activity, including a still unknown PAS domain binding/activating ligand and kinase domain binding modulatory phosphatidylinositol phosphates.

INTRODUCTION

In lower organisms, the PAS (Per-Arnt-Sim)[6] domain is often found in environmental protein sensors involved in the perception of light intensity, oxygen partial pressure, redox potentials, voltage and certain ligands [1]. In mammals, the PAS domain is mainly found as a heterodimerization interface of transcription factors involved in dioxin signalling, the circadian clock and oxygen sensing [2-4]. We and others previously identified a novel mammalian PAS protein, alternatively called PASKIN [5] or PAS kinase [6]. PASKIN contains two PAS domains (PAS A and PAS B) and a serine/threonine kinase domain which might be regulated in *cis* by binding of so far unknown ligands to the PAS domain [7]. PASKIN shows a striking structural similarity to the bacterial oxygen sensor FixL that contains an oxygen-binding heme group within its PAS domain [5]. Following derepression by ligand binding, autophosphorylation in *trans* results in the "switch-on" of the kinase domain of FixL. A similar mode of activation has been suggested for PASKIN, too [6].

PSK1 and PSK2, the budding yeast homologs of PASKIN, phosphorylate three translation factors and two enzymes involved in the regulation of glycogen and trehalose synthesis, thereby coordinately controlling translation and sugar flux [8]. Further experiments revealed that under stress conditions, yeast PSK regulates Ugp1 translocation to the plasma membrane where it increases cell wall glucan synthesis at the expense of glycogen storage. In the absence of PSKs, glycogen rather than glucan is produced, affecting the strength of the cell wall [9]. Two independent cell stressors have been identified to activate PSKs in yeast. Cell integrity stress (e.g. heat shock or SDS treatment) required the Wsc1 membrane stress sensor, and growth in non-glucose carbon sources (e.g. raffinose) required the AMP-dependent kinase (AMPK) homolog Snf1. While PSK2 was predominantly activated by Wsc1, PSK1 was indispensable for Snf1 function [10].

In mammals, PASKIN-dependent phosphorylation inhibits the activity of glycogen synthase (GYS) [11]. PASKIN has also been suggested to be required for glucose-dependent transcriptional induction of preproinsulin gene expression, which might be related to PASKIN-dependent regulation of the nuclear import of pancreatic duodenal homeobox-1 (PDX1) transcription factor [12,13]. However, by generating PASKIN-deficient knock-out mice, we could not demonstrate any PASKIN-dependent difference in insulin gene expression or glucose tolerance [14,15]. Moreover, conflicting data were also reported on the resistance of these *Paskin* knock-out mice towards high fat diet-induced metabolic syndrome [16,17].

We previously found that the eukaryotic translational elongation factor (eEF)1A1 is phosphorylated by PASKIN, probably leading to increased protein translation [18]. By screening for new PASKIN kinase targets, we demonstrate here that another crucial translation factor, ribosomal protein S6, can be phosphorylated by PASKIN, suggesting that

PASKIN regulates protein translation not only in yeast but also in mammals. Moreover, we identified phospholipid ligands binding to PASKIN and studied their effects on PASKIN activity.

RESULTS

Identification of novel PASKIN kinase targets

Two approaches were applied to search for novel mammalian PASKIN targets: yeast two-hybrid and phosphorylation of peptide arrays. By yeast two-hybrid screening of a HeLa cell-derived library, we previously identified eEF1A1 as a PASKIN target [18]. In addition to novel proteins interacting with PASKIN, we also screened for novel proteins which can be phosphorylated by PASKIN. Therefore, a peptide microarray containing 1176 potential phosphoacceptor peptides was incubated with recombinant PASKIN and radioactively labelled ATP. As shown in Fig. 1A, distinct peptides were strongly phosphorylated by PASKIN (see supplemental Table S1 for a list of the 75 most strongly phosphorylated peptides). The consensus phosphoacceptor site of the 30 most strongly phosphorylated peptides was found to be similar to protein kinase (PK) A and C motifs (Fig. 1B). These data are supported by recent findings based on a combinatorial peptide library, demonstrating a strong preference for arginine at position -3 [19]. Accordingly, from the 75 strongest hits in our screening, 70 hits indeed contain arginine three amino acids before the serine or threonine phosphoacceptor site (supplemental table S1). Several proteins were identified more than once either because more than one phosphoacceptor site within the same protein could be phosphorylated, or because overlapping peptides containing the same phosphoacceptor site were present, or because the peptide was derived from the same site but from distinct species. Seventeen different pyruvate kinase-derived peptides, for example, were identified by this way. One of the proteins listed in Fig. 1B is glycogen synthase which has previously been identified by others as a PASKIN kinase target [11]. Thus, glycogen synthase identification confirmed the feasibility of our approach and was used as a reference target protein for the next experiments.

To corroborate PASKIN-dependent phosphorylation of these rather short arrayed peptides, 11 of the most strongly phosphorylated candidate PASKIN kinase targets were synthesized as 20-mer peptides and used for *in vitro* phosphorylation by recombinant PASKIN (see supplemental Table S2 for sequences and references). As shown in Fig. 1C, 6 peptides were significantly better phosphorylated by PASKIN than the unrelated control peptide and 3 of them showed an even stronger phosphorylation than the known PASKIN targets GYS and PDX1, i.e. 40S ribosomal protein S6 (S6), phosphorylase kinase β (PHKB) and phosphofructo kinase/fructose bisphosphatase (PFKFB).

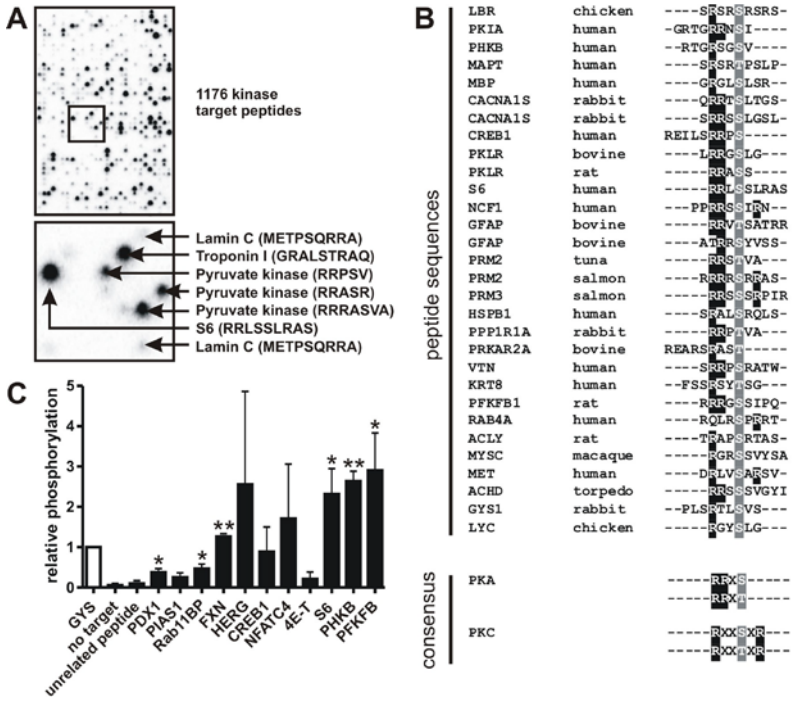


Fig. 1. Identification of novel PASKIN kinase targets. (A) Recombinant His_6-PASKIN purified from SF9 insect cells was used for *in vitro* phosphorylation of a microarray of 1176 peptides in the presence of (γ-^{33}P)-ATP. The magnified inset shows an example of the results following detection by phosphorimaging. (B) Peptide sequences of the 30 most phosphorylated targets and their similarities to PKA and PKC consensus motifs. (C) Target validation. Biotinylated peptides of 20 amino acids length were incubated together with recombinant PASKIN in the presence of (γ-^{33}P)-ATP, captured with streptavidin sepharose beads and quantified by liquid scintillation counting. The sequences were normalised to a GYS-derived peptide, a known target for PASKIN. A PDX-1-derived peptide, another known PASKIN target, served as second positive control. Shown are mean values + SD of n = 3 independent experiments. Asterisks indicate statistically significant differences compared to the unrelated negative control peptide derived from activating transcription factor ATF-4 (*, $p < 0.05$; ** $p < 0.01$; paired t-test). The peptides were named as follows: GYS, glycogen synthase; PDX1, pancreatic and duodenal homeobox 1; PIAS1, protein inhibitor of activated STAT 1; RAB11BP, RAB11-binding protein; FXN, frataxin; HERG, human ether-a-go-go related gene; CREB1, cAMP response element-binding protein; NFATC4; nuclear factor of activated T-cells c4; 4E-T, eIF4E-transporter; S6, 40S ribosomal protein S6; PHKB, phosphorylase kinase β; PFKFB, 6-phosphofructokinase/fructose-2,6-bisphosphatase. Note that some of the PASKIN target sequences as shown in (B) can be found in several distinct proteins, leading to the partially altered designations in (C), as outlined in supplemental Table S2.

Ribosomal protein S6 is phosphorylated by PASKIN

Because others and we previously reported a role for PASKIN in protein translation [8,18], the finding that a S6-derived peptide was strongly phosphorylated by PASKIN was further investigated. S6 is a target of the mTOR signalling pathway that regulates nutrient-dependent protein translation by p70 S6 kinase (p70S6K)-mediated phosphorylation of S6 serine residues 235 and 236 [20]. Therefore, recombinant S6 was expressed and purified either as wild-type, C-terminally truncated or Ser235/236Ala double-mutant GST fusion protein (Fig. 2A). As shown in Fig. 2B, PASKIN phosphorylated wild-type but not truncated or serine double-mutant S6 *in vitro*, suggesting that PASKIN also targets S6 at serines 235/236.

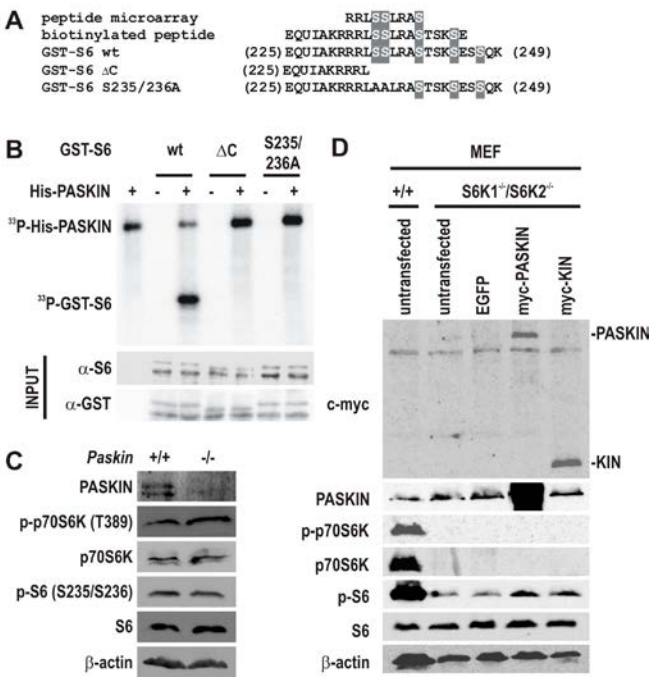


Fig. 2. Ribosomal protein S6 is phosphorylated by PASKIN. (A) Sequence comparison of the S6 peptides used in the microarray, 20-mer peptide used for the *in vitro* reactions, and recombinant GST fusion proteins purified from Sf9 insect cells. (B) Phosphorylation reactions *in vitro* using purified His_6-PASKIN and recombinant S6 in the presence of (γ-^{33}P)-ATP. Following SDS-PAGE, the phosphorylated proteins were visualized by phosphorimaging. Equal input was controlled by immunoblotting against S6 and the GST-tag. (C) Immunoblot analysis of the phosphorylation status of p70S6K and S6 in *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ MEFs. (D) Immunoblot analysis of the phosphorylation status of p70S6K and S6 in S6K1$^{-/-}$/S6K2$^{-/-}$ double knock-out MEFs following overexpression of a negative control (EGFP), myc-PASKIN or myc-KIN. Anti-myc and anti-PASKIN antibodies were used to confirm PASKIN overexpression.

To analyse PASKIN-dependent phosphorylation of endogenous S6 *in vivo*, we used MEFs derived from either *Paskin*$^{+/+}$ wild-type or *Paskin*$^{-/-}$ knock-out mice [14]. However, as shown in Fig. 2C, no difference in constitutive p70S6K or S6 phosphorylation could be detected in these cells. Since basal S6 phosphorylation by p70S6K might overcome subtle changes caused by PASKIN, we next used MEFs deficient for both genes encoding mouse p70S6K (S6K1$^{-/-}$/S6K2$^{-/-}$) [21], and transiently overexpressed full-length PASKIN or an N-terminally truncated version preserving the kinase domain in these cells. Whereas S6 total protein levels remained unchanged, phosphorylated S6 was strongly reduced in S6K1$^{-/-}$/S6K2$^{-/-}$ double knock-out MEFs (Fig. 2D). Interestingly, overexpression of myc-tagged PASKIN, or its kinase domain alone, led to increased phosphorylation of S6 at serines 235/236 (Fig. 2D). In summary, while these data demonstrate that S6 is a new *in vitro* target, PASKIN can also phosphorylate S6 *in vivo* and might even partially contribute to the residual S6 phosphorylation observed in p70S6K-deficient cells [22]. However, a more prominent S6 kinase function *in vivo* probably awaits the identification of the endogenous stimulus of PASKIN catalytic activity.

Autophosphorylation of recombinant PASKIN is activated by phospholipids

A possible mechanism of PASKIN activation *in vivo* might be the binding of a so far unknown ligand like it has been suggested previously [7]. However, no endogenous PASKIN ligand is known so far. By comparing the activity of PASKIN with PKCδ we obtained first hints for a potential endogenous ligand. We previously reported that both PASKIN and PKCδ phosphorylate the eukaryotic translation elongation factor eEF1A1 [18], and both kinases are known to autophosphorylate themselves [6,23]. Because PKCδ kinase activity is known to be stimulated by diacylglycerol (DAG) and phosphatidylserine (PS) [24], we wondered whether other similarities exist between PASKIN and PKCδ. Interestingly, a mixture of PS and DAG (used in the form of dioctanoylglycerol, DOG) not only enhanced PKCδ but also PASKIN autophosphorylation (Fig. 3A).

In order to systematically analyze the lipid activation of PASKIN, all major phospholipids were compared for their effects on PASKIN and PKCδ autophosphorylation. As shown in Fig. 3B, all tested phospholipids but not DOG alone increased PASKIN autophosphorylation. In contrast, PKCδ autophosphorylation was induced by DOG alone, to some extent also by PS or PC, but all other phospholipids had only marginal effects on PKCδ. As shown before [24], a mixture between DOG and PS was required to maximally induce PKCδ activity. However, combining DOG with phospholipids did not further induce PASKIN (data not shown).

The rather unselective stimulation of PASKIN activity by all tested phospholipids suggested that the core phospholipid moiety might confer PASKIN binding. Indeed, as shown in Fig. 3C phosphatidic acid alone was sufficient to stimulate PASKIN autophosphorylation. The finding that PA but not DOG strongly bound PASKIN suggested that PLD might target PASKIN by converting phospholipids into PA. To directly demonstrate this assumption, 96-well plates were coated with constant amounts (1 μg) of PC. Following treatment with phospholipase (PL) D or C, increasing amounts of PASKIN were added and detected by ELISA. As shown in Fig. 3D, PASKIN bound with clearly higher affinity to PA than to PC. However, lipid binding was restored when PC was treated with PLD (generating PA) but not with PLC (generating DAG).

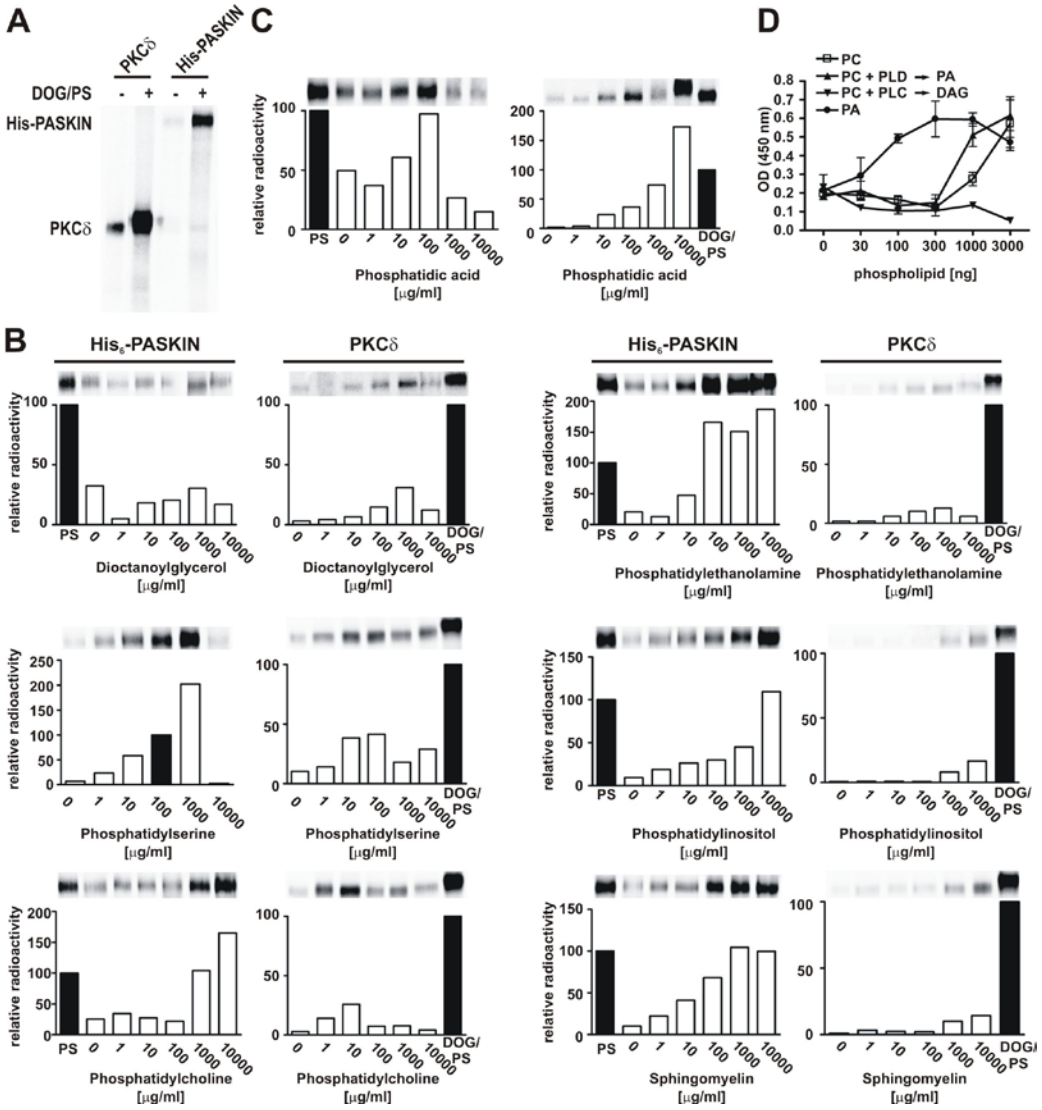


Fig. 3. Phospholipid stimulation of PASKIN autophosphorylation. (A) Lipid stimulation of PKCδ and PASKIN autophosporylation as assessed by incubating the purified recombinant proteins with DOG/PS mixtures and (γ-^{33}P)-ATP. Following SDS-PAGE, the phosphorylated proteins were visualized by phosphorimaging. (B, C) Stimulation of PASKIN and PKCδ autophosphorylation by increasing amounts of the indicated phospholipids. Following SDS-PAGE, the phosphorylated proteins were visualized (upper panels) and quantified (lower panels) by phosphorimaging. The values were normalised to 100 μg/ml PS and 10 μg/ml DOG/100 μg/ml PS mixtures for PASKIN and PKCδ, respectively (filled columns). (D) PLD but not PLC converts PC from a low affinity to a high affinity PASKIN ligand. 96-well plates were coated with increasing amounts of PC, followed by treatment with PLD or PLC as indicated. Binding of 100 ng PASKIN added to each well was detected by ELISA. Shown are mean values ± SD of a representative experiment performed in triplicates.

Inositol phosphorylation determines the affinity of phospatidylinositol interaction with PASKIN

The experiments described above suggested that an isolated phosphate group such as in PA is necessary for maximal PASKIN-lipid interaction. Because phosphatidylinositols (PtdIns) with varying numbers of phosphate groups belong to the most important cellular lipid signalling molecules, we next investigated whether the number and location of the phosphate groups on the inositol ring affect their interaction with PASKIN. Therefore, dot blots containing mono-,

di- and tri-phosphorylated PtdIns were incubated with recombinant PASKIN and immunodetected using anti-PASKIN antibodies. Surprisingly, whereas unphosphorylated PI showed only relatively low PASKIN binding, this interaction was strongly increased by the presence of a single phosphate group in PtdIns(4)P, and reduced again when two or three phosphate groups were present in PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, respectively (Fig. 4A). This finding was corroborated by using dot blots with increasing amounts of all possible PtdIns-phosphates: PASKIN dose-dependently bound better PtdIns-monophosphates than PtdIns-diphosphates, and non-phosphorylated or tri-phosphorylated PtdIns bound PASKIN only weakly (Fig. 4B, left panel). Similar results were obtained with autophosphorylated PASKIN (Fig. 4B, right panel), suggesting that PASKIN phosphorylation status does not interfere with selective PtdIns-monophosphate binding.

To localise the region responsible for PtdIns-monophosphate binding, four different fragments of PASKIN as indicated in Fig. 4C (left panel) were expressed and purified as His_6-tagged fusion proteins. However, only the kinase (KIN) domain of PASKIN bound PtdIns-monophosphates (Fig. 4C, right panel), rather than the previously suggested ligand-binding PAS domain (data not shown). We next sought to determine the effects of differently phosphorylated PtdIns on PASKIN autophosphorylation. As shown in Fig. 4D, autophosphorylation was dose-dependently enhanced by all three PtdIns-monophosphates, whereas especially high concentrations of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 even inhibited autophosphorylation, establishing a structure-function relationship between kinase domain-lipid interaction and kinase activity.

PtdIns-monophosphate-dependent regulation of PASKIN target phosphorylation

Because PtdIns-monophosphates stimulated PASKIN autophosphorylation, we wondered whether they could also stimulate phosphorylation of the PASKIN targets S6 and eEF1A1. Therefore, wild-type and phosphoacceptor site mutant recombinant S6 and eEF1A1 were used for PASKIN *in vitro* phosphorylation reactions in the presence of differently phosphorylated PtdIns-phosphates. As shown in Fig. 5, PASKIN auto-phosphorylation was again stimulated by all three PtdIns-monophosphates but inhibited by PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. While the phosphoacceptor site mutant S6 and eEF1A1 GST fusion proteins remained unphosphorylated, their wild-tpye counterparts were phosphorylated by PASKIN. Unexpectedly, both S6 and eEF1A1 target phosphorylation was inhibited by PtdIns-phosphates. The more phosphate groups the inositol ring carries, the stronger was PASKIN target protein phosphorylation inhibited. However, non-phosphorylated PtdIns did not significantly change target phosphorylation efficiency.

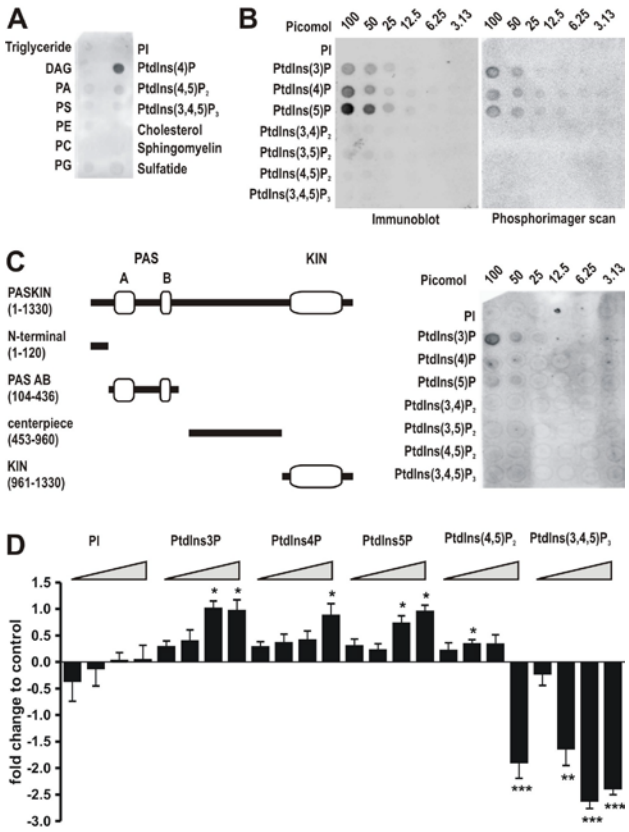


Fig. 4. Preferential PASKIN binding to, and activation by, phosphatidylinositol monophosphates. (A) Recombinant His_6-PASKIN protein was allowed to bind to the indicated lipids immobilised on a membrane, and subsequently detected using an anti-PASKIN antibody. (B) PASKIN dose-dependently bound preferably phospatidylinositol (PtdIns) monophosphates. PASKIN was either detected by immunoblotting (left panel) or by phosphorimaging following autophosphorylation in the presence of (γ-^{33}P)-ATP (right panel). (C) Fragments of PASKIN were expressed in *E. coli* and purified as His_6-tagged fusion proteins (left panel). Following binding to the lipid dot blots and detection using a His-tag antibody, only the kinase (KIN) domain of PASKIN was found to interact with PtdIns-monophosphates (right panel). (D) His_6-PASKIN autophosphorylation was mainly stimulated by the presence of the PtdIns-monophosphates. *In vitro* phosphorylation reactions in the presence of (γ-^{33}P)-ATP and the indicated synthetic diC8 PtdIns (3.16 μM, 10 μM, 31.6 μM and 100 μM) were separated by SDS-PAGE and quantified by phosphorimaging. Values were expressed relative to lipid-free control reactions and are represented as mean + SD of n = 4 independent experiments (*, $p < 0.05$; ** $p < 0.01$; ***, $p < 0.001$; t-test).

DISCUSSION

In this work, we identified various novel potential PASKIN substrates by peptide microarray phosphorylation, including glycogen synthase that was known before to be phosphorylated by PASKIN [11]. Thus, the repetitive identification of this PASKIN target confirms, at least partially, the validity of the peptide array approach. Other peptides derived from proteins involved in glycogen metabolism included phosphorylase kinase, inhibitor of protein phosphatase 1 and yeast glycogen phosphorylase (supplemental Table S1). The involvement of PASKIN in the regulation of glycogen synthesis has previously been demonstrated by showing that both mammalian and yeast glycogen synthases as well as yeast UDP-glucose pyrophosphorylase are known phosphorylation targets of mammlian PASKIN and yeast

PSK1 and PSK2, respectively [8,11]. However, whereas Ser640 was the main PASKIN kinase target residue of mammalian glycogen synthase [11], the peptides phosphorylated by PASKIN on the microarray contained Ser3 and Ser7 but not Ser640. Of note, a Ser640Ala mutation did not completely prevent phosphorylation [11]. Therefore, our data suggest that PASKIN might phosphorylate Ser3 and/or Ser7 of glycogen synthase in addition to Ser640.

Two peptides phosphorylated by PASKIN were derived from enzymes involved in glycolysis: pyruvate kinase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1 (PFKFB1) (supplemental Table S1). Obviously, the coordination of glycolysis, gluconeogenesis and glycogen synthesis appears to be physiologically meaningful, and it is hence tempting to speculate that PASKIN is involved in the regulation of all of these metabolic pathways. However, pyruvate kinase could not be confirmed as a PASKIN target using purified full-length pyruvate kinase GST fusion proteins in *in vitro* assays (data not shown).

Interestingly, S6 was among the peptides phosphorylated by PASKIN and this phosphorylation could be confirmed on the full-length protein level. Together with the previously reported eEF1A1 phosphorylation [18], this finding provides additional evidence that PASKIN is involved in mammalian protein translation. The most important and best characterised S6 kinases are the mTOR dependent p70 S6-kinases that sequentially phosphorylate all five phosphorylatable serines of S6, starting with S236 and S235 - the same sites as shown herein for PASKIN - followed by S240, S244 and S247 [25]. A second family of S6 kinases are p90 ribosomal S6 kinases (RSK) that phosphorylate S6 upon mitogenic stimulation at the same sites as PASKIN [22]. Phosphorylation of S6 by p70S6K has long been thought to increase protein translation by selectively enhancing the translation of 5'-TOP mRNAs, a subset of mRNAs containing an oligopyrimidine tract in their 5'-untranslated regions. Of note, the 5'-TOP mRNAs code for ribosomal proteins and translation factors including PASKIN targets S6 and eEF1A1 [26]. However, S6 phosphorylation and increased 5'-TOP mRNA translation might be coincidental rather than causally related [27], and, according to a newer hypothesis, might even negatively influence translation if phosphorylation of S6 is considered an inhibitory feed-back signal [28]. However, no significant difference in global (^{35}S)-Met incorporation could be observed in *Paskin*$^{-/-}$ MEFs (data not shown).

Based on the known functions of the PASKIN-related FixL oxygen sensor in bacteria and the PASKIN orthologs in yeast, and considering the lack of any obvious phenotype in *Paskin* knock-out mice kept under normal housing conditions, it is tempting to assume that PASKIN has a ligand-mediated sensor function that becomes apparent under currently ill defined stress situations [17]. However, only artificial but no endogenous PASKIN ligands have been reported up to now to bind the PAS domain and lead to the de-repression of the

kinase domain dependent autophosphorylation [7]. Here, we identified phospholipids as the first biologically relevant PASKIN ligands. Apparently, the presence of a charged phosphate moiety is required for stimulation of PASKIN kinase activity, and PLD, but not PLC, can convert phospholipids from low into high-affinity PASKIN ligands. However, we currently do not know whether PASKIN is a target of intracellular PLD cell signalling.

Surprisingly, PtdIns-monophosphates were found to be the best ligands of PASKIN with clearly higher affinities than PtdIns-diphosphates or PtdIns-triphosphate. PtdIns binding domains have been reported to display either well-defined three dimensional folds [29], or rather unstructured regions with basic (for binding of the phosphate groups) and hydrophobic residues, like in the non-canonical pleckstrin homology (PH) domain of Tiam1 [30]. We identified a lysine rich region, spanning from Lys1019 to Lys1034 of PASKIN, which shares characteristic features with non-canonical PH domains, including a double lysine motif (Lys1031/1032). However, mutation and deletion analyses of this putative binding region did not affect lipid binding by PASKIN (data not shown). Thus, it is difficult to predict the PtdIns-monophosphate binding site within the PASKIN kinase domain and further work will be necessary to identify the specific residues involved in lipid binding.

Whereas PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are involved in signalling processes at the plasma membrane, PtdIns-monophosphates are more abundant in intracellular membrane structures such as the Golgi apparatus and endosomes [31]. Within these structures, PtdIns-monophosphates are involved in sorting and signalling, since the concentrations and localization of differentially phosphorylated PtdIns can change rapidly [29]. Therefore, it might be possible that PtdIns-monophosphates not only regulate PASKIN activity but also its subcellular localization. This hypothesis needs further investigations but is dependent on the prior identification of the specific environmental conditions that regulate PASKIN function.

As could have been expected, we found a direct correlation between ligand affinity and PASKIN autophosphorylation efficiency. However, it turned out to be the kinase domain rather than the PAS domain which binds the PtdIns-phosphates. This finding might explain why the activation of PASKIN-dependent S6 and eEF1A1 target phosphorylation failed to comply with our initial expectations: PASKIN autophosphorylation was not directly related to target phosphorylation. However, our results are consistent with a recent report demonstrating that PASKIN kinase activity is independent of activation loop phosphorylation [19]. Thus, the original model of autophosphorylation-dependent kinase activity needs to be revised, and the functional meaning of PASKIN autophosphorylation remains to be elucidated.

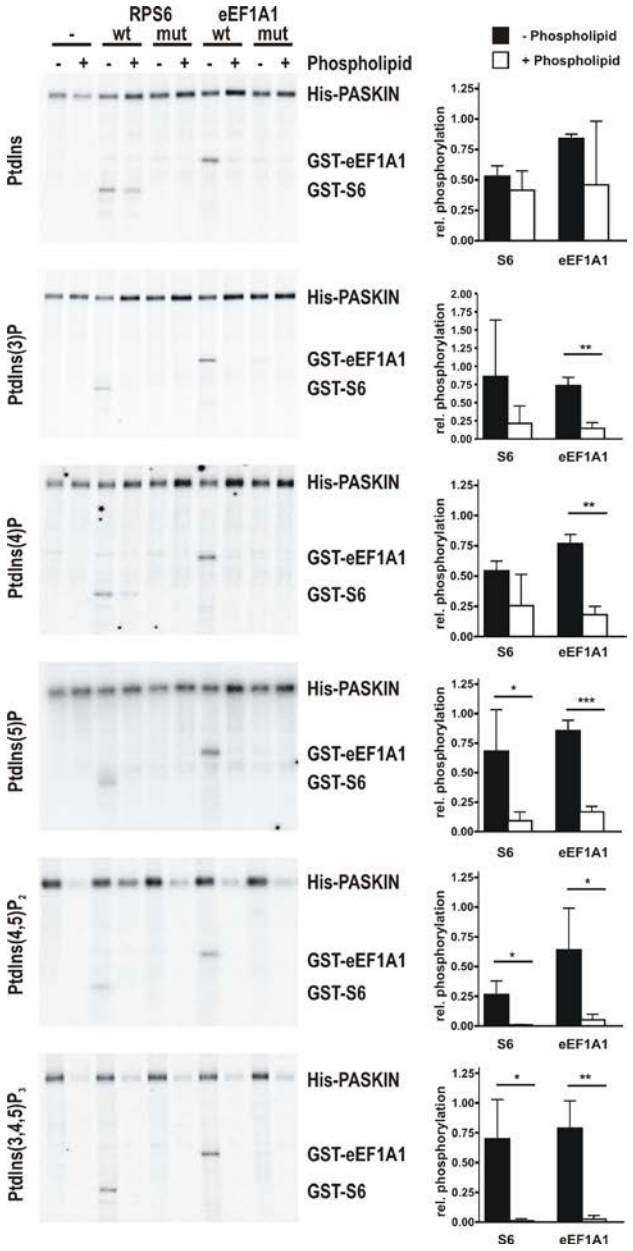


Fig. 5. *In vitro* target phosphorylation of His_6-PASKIN is reduced in presence of phosphatidylinositides. Recombinant His_6-PASKIN purified from Sf9 insect cells was used to *in vitro* phosphorylate recombinant GST fusion proteins with wild-type S6, with the non-phosphorylatable double mutant S235/236A, with eEF1A1, or with its non-phosphorylatable T432A mutant, in the presence of (γ-^{33}P)-ATP and PtdIns phosphates (100 μM) as indicated. Following separation by SDS-PAGE, protein phosphorylation was viusalized (left panel, representative images) and quantified (right panel) by phosphorimaging. His_6-PASKIN autophosphorylation without lipid and target (first lane from the left) was used for intra-assay normalisation of the values. Columns represent mean values + SD of n = 3 independent experiments (*, $p < 0.05$; ** $p < 0.01$; ***, $p < 0.001$; t-test).

In conclusion, our *in vitro* data suggest similar downstream effector functions of mammalian PASKIN as known from yeast: the coordination between energy flux and translation. With the identification of endogenous small molecule activators of PASKIN, we obtained the first hints on the upstream regulators of PASKIN activity and it will be interesting to examine how these regulators affect the downstream processes mediated by PASKIN.

EXPERIMENTAL PROCEDURES

Plasmids

All cloning work was carried out using Gateway technology (Invitrogen). The human PASKIN cDNA containing plasmids pENTR4-hPASK and pENTR4-hKIN, and plasmids for recombinant expression of full length His_6-PASKIN, PASKIN truncations and eukaryotic elongation factor 1A1 have been published previously [18]. pENTR4-hPASK and pENTR4-hKIN were recombined into pcDNA3.1/c-myc-DEST [32] using LR recombinase (Invitrogen) to generate pcDNA3.1/c-myc-hPASK and pcDNA3.1/c-myc-hKIN for c-myc-tagged expression of PASKIN or its kinase domain, respectively, in mammalian cells. Human ribosomal protein S6 (IRAUp969B0849D6, Deutsches Ressourcenzentrum für Genomforschung) was cloned into pENTR4 using forward and reverse primers 5'-ttatgtcgacatgaagctgaacat-3' and 5'-tacgtgcggccgcttatttctgactggattcagacttag-3', respectively, or 5'-tacgtggcggccgcttaaagtctgcgtctcttcgc-3' to introduce a stop codon after residue L234. The PCR products were ligated into the SalI and NotI restriction sites. The S6 S235/236A double mutant was produced with primers 5'-gcgaagagacgcaggctagccgctctgcgagcttctac-3' and 5'-gtagaagctcgcagagcggctagcctgcgtctcttcgc-3' by Pfu polymerase-based site-directed mutagenesis (Stratagene). For expression as GST-tagged fusion proteins, pENTR4 based plasmids with the different S6 constructs were recombined into pDEST15 using LR recombinase.

Purification of recombinant proteins

Recombinant proteins were purified as described in detail previously [18]. Briefly, full-length PASKIN was purified from Sf9 cells using the Bac-to-Bac Baculovirus expression system (Invitrogen). GST-tagged fusion constructs and His_6-tagged PASKIN fragments were expressed in arabinose inducible BL21 *E. coli*. Recombinant proteins were purified by FPLC (BioLogic DuoFlow, Biorad) using HiTrap Chelating HP and GSTrap FF columns (GE Healthcare), respectively. The kinase activity of purified recombinant PASKIN was verified by auto-phosphorylation assays.

Kinase assays

His_6-PASKIN or PKCδ (Invitrogen) were incubated with or without 2 μg recombinant target proteins in kinase buffer (25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) for 20 minutes in the presence of 3 μCi (γ-^{33}P)ATP (Hartmann Analytic). Proteins were separated by SDS-PAGE and analyzed by phosphorimaging of the dried gels (Molecular Imager FX, Bio-Rad) using Quantity One software (Bio-Rad). Lipids (Sigma or Fluka) were dissolved in

$CHCl_3$, aliquotted in test tubes and the $CHCl_3$ evaporated under a stream of nitrogen. Lipids were then resuspended in kinase assay master mixes by thorough vortexing. Phosphatidylinositides present in the phosphorylation reactions were obtained from Echelon Biosciences as synthetic diC8-lipids and added to the reactions from 1 mM aequous stock solutions to the final concentrations indicated.

Peptide microarrays

Peptide microarrays were phosphorylated with recombinant PASKIN according to the manufacturer's instructions (Pepscan). In brief, 50 µl of a solution containing 500 ng recombinant PASKIN, 50 mM HEPES pH 7.4, 20 mM $MgCl_2$, 10% glycerol, 300 µCi/ml (γ-^{33}P)ATP, 0.01% (v/v) Brij-35 and 0.01 mg/ml BSA was added to the glass slide, covered with a glass coverslip and incubated at 30°C for 2 hours in a humidified incubator. After incubation, the coverslip was removed with 1% Triton X-100 in PBS and the glass slide was washed twice with 1% Triton X-100 in 2 M NaCl and twice with water by over-head shaking, air-dried and analyzed by phosphorimaging (Bio-Rad).

Phosphorylation of biotinylated peptides

PASKIN phosphorylation reactions were performed as described above in the presence of N-terminally biotinylated 20-mer target peptides (JPT peptide technologies) at 200 µM final concentration. The reactions were stopped by adding SDS to 0.5% final concentration and heating at 95°C for 5 minutes. Streptavidin sepharose beads (25 µl, GE Healthcare) and 500 µl of 100 mM Tris-HCl pH 8.0 were added and incubated for 30 minutes at 4°C. The beads were washed three times with 500 µl of a buffer containing 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 400 mM NaCl, 0.1% Nonidet P-40 and once with 500 µl 100 mM Tris pH 8.0. Phosphorylation of the beads was quantified by liquid scintillation counting (Tri-Carb 2900TR, Packard-Perkin Elmer).

Cell culture, transfections and immunoblotting

Mouse embryonic fibroblast (MEF) cells were generated from *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice [14] at embryonic day 14 (E14). *S6K1*$^{-/-}$/*S6K2*$^{-/-}$ double knock-out MEFs were kindly provided by G. Thomas and S. C. Kozma (Basel, Switzerland). MEF cells were cultivated in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% FCS (Invitrogen) up to passage 12, implying that they immortalised spontaneously. MEFs were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. 36 hours post transfection, cells were harvested and whole cell lysates were generated by heating the cells in 1% SDS for 5 minutes at 95°C. After SDS-PAGE and

immunoblotting, the following primary antibodies were used: human PASKIN (Affinity Bioreagents); mouse PASKIN, phospho-S6 (S235/236), S6 kinase and phospho-S6 kinase (T389) (Cell Signaling); S6 (Bethyl); β-actin and GST-tag (Sigma); His-tag (Novagen).

Lipid binding assays

Interactions between PASKIN and lipids were measured by an ELISA-based assay described previously [33]. Briefly, 96-well plates (Sarstedt) were coated overnight with phospholipids dissolved in methanol, followed by blocking with 3% BSA in PBS for 1 hour. Purified His_6-PASKIN (100 ng) was diluted in kinase buffer and allowed to bind for 1 hour at 30°C. After three washing steps (0.3% Tween-20 in PBS), bound PASKIN was detected by anti-PASKIN mAb6, followed by secondary goat anti-mouse HRP-conjugated antibodies (Pierce) using the 3,3',5,5'-tetramethylbenzidine substrate kit (Pierce). The peroxidase reaction was stopped by adding H_2SO_4 (1 M final concentration) and absorbance was determined at 450 nm in a microplate reader (Digiscan, Asys Hitech). For phospholipase (PL) experiments, phospholipid-coated 96-well plates were treated with 0.2 units PLC or PLD (Sigma), diluted in reaction buffer (120 mM $CaCl_2$, 300 mM sodium acetate pH 5.6) for 1 hour at room temperature.

Lipid binding arrays

Membranes spotted with phospholipids were obtained from Echelon Biosciences (P-6002, P-6100) and used according to the manufacturer's protocol. Generally, 1 μg of protein diluted in 1% skimmed dry milk in TBS was allowed to bind to spotted phospholipids for 16-20 hours at 4°C. Binding was detected using primary antibodies as indicated and HRP-coupled secondary antibodies for enhanced chemiluminescence detection (Pierce).

ACKNOWLEDGEMENTS

The authors wish to thank J. Rutter, G. Thomas and S. C. Kozma for the generous gifts of plasmids and cell lines; and Gieri Camenisch and Daniel P. Stiehl for helpful discussions. This work was supported by by grants from the Wolfermann-Nägeli Stiftung, Stiftung für wissenschaftliche Forschung and der Universität Zürich/Baugarten Stiftung, the University Research Priority Program "Integrative Human Physiology", and the Swiss National Science Foundation (Grant SNF 31003A_129962/1).

REFERENCES

1 Ponting CP & Aravind L (1997) PAS: a multifunctional domain family comes to light. *Curr Biol* **7**, R 674-R 677.

2 Lowrey PL & Takahashi JS (2000) Genetics of the mammalian circadian system: Photic entrainment, circadian pacemaker mechanisms, and posttranslational regulation. *Annu Rev Genet* **34**, 533-562.

3 Mimura J & Fujii-Kuriyama Y (2003) Functional role of AhR in the expression of toxic effects by TCDD. *Biochim Biophys Acta* **1619**, 263-268.

4 Wenger RH (2002) Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression. *FASEB J* **16**, 1151-1162.

5 Hofer T, Spielmann P, Stengel P, Stier B, Katschinski DM, Desbaillets I, Gassmann M & Wenger RH (2001) Mammalian PASKIN, a PAS-serine/threonine kinase related to bacterial oxygen sensors. *Biochem Biophys Res Commun* **288**, 757-764.

6 Rutter J, Michnoff CH, Harper SM, Gardner KH & McKnight SL (2001) PAS kinase: An evolutionarily conserved PAS domain-regulated serine/threonine kinase. *Proc Natl Acad Sci USA* **98**, 8991-8996.

7 Amezcua CA, Harper SM, Rutter J & Gardner KH (2002) Structure and interactions of PAS kinase N-terminal PAS domain: model for intramolecular kinase regulation. *Structure* **10**, 1349-1361.

8 Rutter J, Probst BL & McKnight SL (2002) Coordinate regulation of sugar flux and translation by PAS kinase. *Cell* **111**, 17-28.

9 Smith TL & Rutter J (2007) Regulation of glucose partitioning by PAS kinase and Ugp1 phosphorylation. *Mol Cell* **26**, 491-499.

10 Grose JH, Smith TL, Sabic H & Rutter J (2007) Yeast PAS kinase coordinates glucose partitioning in response to metabolic and cell integrity signaling. *EMBO J* **26**, 4824-4830.

11 Wilson WA, Skurat AV, Probst B, de Paoli-Roach A, Roach PJ & Rutter J (2005) Control of mammalian glycogen synthase by PAS kinase. *Proc Natl Acad Sci USA* **102**, 16596-16601.

12 da Silva Xavier G, Rutter J & Rutter GA (2004) Involvement of Per-Arnt-Sim (PAS) kinase in the stimulation of preproinsulin and pancreatic duodenum homeobox 1 gene expression by glucose. *Proc Natl Acad Sci USA* **101**, 8319-8324.

13 An R, da Silva Xavier G, Hao HX, Semplici F, Rutter J & Rutter GA (2006) Regulation by Per-Arnt-Sim (PAS) kinase of pancreatic duodenal homeobox-1 nuclear import in pancreatic beta-cells. *Biochem Soc Trans* **34**, 791-793.

14 Katschinski DM, Marti HH, Wagner KF, Shibata J, Eckhardt K, Martin F, Depping R, Paasch U, Gassmann M, Ledermann B, Desbaillets I & Wenger RH (2003) Targeted disruption of the mouse PAS domain serine/threonine kinase PASKIN. *Mol Cell Biol* **23**, 6780-6789.

15 Borter E, Niessen M, Zuellig R, Spinas GA, Spielmann P, Camenisch G & Wenger RH (2007) Glucose-stimulated insulin production in mice deficient for the PAS kinase PASKIN. *Diabetes* **56**, 113-117.

16 Hao HX, Cardon CM, Swiatek W, Cooksey RC, Smith TL, Wilde J, Boudina S, Abel ED, McClain DA & Rutter J (2007) PAS kinase is required for normal cellular energy balance. *Proc Natl Acad Sci USA* **104**, 15466-15471.

17 Schläfli P, Borter E, Spielmann P & Wenger RH (2009) The PAS-domain kinase PASKIN: a new sensor in energy homeostasis. *Cell Mol Life Sci* **66**, 876-883.

18 Eckhardt K, Tröger J, Reissmann J, Katschinski DM, Wagner KF, Stengel P, Paasch U, Hunziker P, Borter E, Barth S, Schläfli P, Spielmann P, Stiehl DP, Camenisch G & Wenger RH (2007) Male germ cell expression of the PAS domain kinase PASKIN and its novel target eukaryotic translation elongation factor eEF1A1. *Cell Physiol Biochem* **20**, 227-240.

19 Kikani CK, Antonysamy SA, Bonanno JB, Romero R, Zhang FF, Russell M, Gheyi T, Iizuka M, Emtage S, Sauder JM, Turk BE, Burley SK & Rutter J (2010) Structural bases of PAS domain-regulated kinase (PASK) activation in the absence of activation loop phosphorylation. *J Biol Chem* **285**, 41034-41043.

20 Ferrari S, Bandi HR, Hofsteenge J, Bussian BM & Thomas G (1991) Mitogen-activated 70K S6 kinase. Identification of in vitro 40 S ribosomal S6 phosphorylation sites. *J Biol Chem* **266**, 22770-22775.

21 Pende M, Um SH, Mieulet V, Sticker M, Goss VL, Mestan J, Mueller M, Fumagalli S, Kozma SC & Thomas G (2004) S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol* **24**, 3112-3124.

22 Roux PP, Shahbazian D, Vu H, Holz MK, Cohen MS, Taunton J, Sonenberg N & Blenis J (2007) RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *J Biol Chem* **282**, 14056-14064.

23 Kielbassa K, Müller HJ, Meyer HE, Marks F & Gschwendt M (1995) Protein kinase Cδ-specific phosphorylation of the elongation factor eEF-α and an eEF-1α peptide at threonine 431. *J Biol Chem* **270**, 6156-6162.

24 Bell RM & Burns DJ (1991) Lipid activation of protein kinase C. *J Biol Chem* **266**, 4661-4664.

25 Krieg J, Hofsteenge J & Thomas G (1988) Identification of the 40 S ribosomal protein S6 phosphorylation sites induced by cycloheximide. *J Biol Chem* **263**, 11473-11477.

26 Meyuhas O (2000) Synthesis of the translational apparatus is regulated at the translational level. *Eur J Biochem* **267**, 6321-6330.

27 Ruvinsky I, Sharon N, Lerer T, Cohen H, Stolovich-Rain M, Nir T, Dor Y, Zisman P & Meyuhas O (2005) Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev* **19**, 2199-2211.

28 Meyuhas O (2008) Physiological roles of ribosomal protein S6: one of its kind. *Int Rev Cell Mol Biol* **268**, 1-37.

29 Lemmon MA (2008) Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol* **9**, 99-111.

30 Ceccarelli DF, Blasutig IM, Goudreault M, Li Z, Ruston J, Pawson T & Sicheri F (2007) Non-canonical interaction of phosphoinositides with pleckstrin homology domains of Tiam1 and ArhGAP9. *J Biol Chem* **282**, 13864-13874.

31 Di Paolo G & De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**, 651-657.

32 Barth S, Nesper J, Hasgall PA, Wirthner R, Nytko KJ, Edlich F, Katschinski DM, Stiehl DP, Wenger RH & Camenisch G (2007) The peptidyl prolyl *cis*/*trans* isomerase FKBP38 determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability. *Mol Cell Biol* **27**, 3758-3768.

33 Ghosh S, Strum JC, Sciorra VA, Daniel L & Bell RM (1996) Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-O-tetradecanoylphorbol-13-acetate-stimulated Madin-Darby canine kidney cells. *J Biol Chem* **271**, 8472-8480.

Target Protein	Gene Symbol	Species	Swissprot	Sequence	Length	Phosphosite	Known kinase	Radioactivity
Lamin B receptor	LBR	chicken	P23913	SRSRSRSRS	9	S-80	SRPK1 / LBRK	**14628.8**
Lamin B receptor	LBR	chicken		SRSRSRSPG	9	S-82	SRPK1 / LBRK	**3816.9**
Lamin B receptor	LBR	chicken		RRSRSRSRS	9	S-78	SRPK1 / LBRK	**2472.0**
Peptide derived from cAMP dependent Protein Kinase Inhibitor alpha (14-22aa)	PKIA	human	P61925	GRTGRRNSI	9	S-8	/	**14221.6**
Phosphorylase b kinase β regulatory chain	PHKB	human	Q93100	RTGRSGSV	8	S-26 (K24G)	PKA	**10948.7**
Phosphorylase b kinase β regulatory chain	PHKB	human		RTKRSGSV	8	S-26	PKA	**5074.4**
Phosphorylase b kinase beta regulatory chain	PHKB	rabbit	P12798	RSKRSGSV	8	S-26 (T23S)	PKA	**4953.5**
Phosphorylase b kinase beta regulatory chain	PHKB	rabbit		ARTKRSGSV	9	S-26	PKA	**3528.0**
Phosphorylase b kinase beta regulatory chain	PHKB	rabbit		RTKRSGSV	8	S-26	PKA	**2856.0**
Phosphorylase b kinase beta regulatory chain	PHKB	rabbit		RAKRSGSV	8	S-26 (T23A)	PKA	**2058.5**
Microtubule-associated protein TAU	MAPT	human	P10636	SRSRTPSLP	9	T-212	GSK3	**10459.9**
Myelin Basic Protein	MBP	bovine	P02687	GRGLSLSR	8	S-136	/	**10191.0**
Voltage-dependent L-type calcium channel alpha-1S subunit	CACNA1S	rabbit	P07293	SRRSSLGSL	9	S-1854	PKA	**8368.0**
Voltage-dependent L-type calcium channel alpha-1S subunit	CACNA1S	rabbit		QRRTSLTGS	9	S-1757	PKA	**7337.6**
cAMP response element binding protein	CREB1	human	P16220	REILSRRPS	9	S-129	GSK3	**8292.6**
Pyruvate kinase isozyme L	PKLR	bovine	/	RRASLG	6	S-12	PKA	**6524.6**
Pyruvate kinase isozyme L	PKLR	bovine		LRRGSLG	7	S-12 (A11G; A14G)	PKA	**5140.2**
Pyruvate kinase isozyme L	PKLR	bovine		LRRASLG	7	S-12	PKA	**4409.3**
Pyruvate kinase isozyme L	PKLR	bovine		LRRATLG	7	S-12 (S12T)	PKA	**3538.9**
Pyruvate kinase isozyme L	PKLR	bovine		LRRPSLG	7	S-12 (A11P)	PKA	**2409.1**
Pyruvate kinase isozyme L	PKLR	bovine		LRRASLAG	8	S-12 (G14A)	PKA	**2347.2**
Pyruvate kinase isozyme L	PKLR	bovine		LRRASLRG	8	S-12 (G14R)	PKA	**2205.1**
Pyruvate kinase isozymes R/L	PKLR	rat	P12928	RRASS	5	S12/S-43 (V44S)	PKA	**6374.1**
Pyruvate kinase isozymes R/L	PKLR	rat		RRGSV	5	S12/S-43 (A42G))	PKA	**5923.5**
Pyruvate kinase isozymes R/L	PKLR	rat		RRATVA	6	S12/S-43 (S43T)	PKA	**5449.2**
Pyruvate kinase isozymes R/L	PKLR	rat		RRVSV	5	S12/S-43 (A42V)	PKA	**5410.7**
Pyruvate kinase isozymes R/L	PKLR	rat		RRRRAASVA	9		PKA	**5166.8**
Pyruvate kinase isozymes R/L	PKLR	rat		RRASL	5	S12/S-43 (V44L)	PKA	**5082.9**
Pyruvate kinase isozymes R/L	PKLR	rat		RRRASVA	7		PKA	**4982.9**
Pyruvate kinase isozymes R/L	PKLR	rat		RRRRPTPA	8		PKA	**4964.1**
Pyruvate kinase isozymes R/L	PKLR	rat		RRRRSVA	7	S12/S-43 (L39R; A42R)	PKA	**4591.3**
Pyruvate kinase isozymes R/L	PKLR	rat		RRFSV	5	S12/S-43 (A42F)	PKA	**3823.5**
Pyruvate kinase isozymes R/L	PKLR	rat		RRASI	5	S12/S-43 (V44I)	PKA	**3464.9**
Pyruvate kinase isozymes R/L	PKLR	rat		RRRRASVA	8	S12/S-43 (Y38R; L39R)	PKA	**2645.2**
Pyruvate kinase isozymes R/L	PKLR	rat		RRAASVA	7	S12/S-43 (L39R; R41A)	PKA	**2341.2**
Pyruvate kinase isozymes R/L	PKLR	rat		RRASR	5	S12/S-43 (V44R)	PKA	**2182.3**
Pyruvate kinase isozymes R/L	PKLR	rat		LRRASVA	7	S12/S-43	PKA	**2170.1**
Pyruvate kinase isozymes R/L	PKLR	rat		RRASVA	6	S12/S-44	PKA	**2079.1**
Pyruvate kinase isozymes R/L	PKLR	rat		RRPSV	5	S12/S43 (A42P)	PKA	**2028.6**
40S ribosomal protein S6	RPS6	human	P62753	RRLSSLRAS	9	S-236	S6K	**6258.7**
Neutrophil cytosol factor 1	NCF1	human	P14598	PPRRSSIRN	9	S-303	/	**5853.3**
Glial fibrillary acidic protein	GFAP	bovine	Q28115	RRVTSATRR	9	S-8	PKA	**5674.0**
Glial fibrillary acidic protein	GFAP	bovine	Q28115	ATRRSYVSS	9	S-13	PKA	**3168.5**
Protamine Z1and Z2	PRM2	tuna	P02322	RRSTVA	6	T-21	PKA	**5571.2**
Protamine Z1 and Z2	PRM2	salmon (clupa)	P02336	RRRRSRRAS	9	S-6	PKA and PKC	**4681.7**
Protamine YI	PRM3	salmon (clupa)	P02337	RRSSSRPIR	9	S-8	PKA and PKC	**4905.9**
Heat shock protein 27	HSPB1	human	P04792	SRALSRQLS	9	S-78	MAPKAPK2	**5046.3**
Inhibitor of protein phosphatase 1	PPP1R1A	rabbit	P01099	RRPTVA	6	T-35	PKA	**4772.6**
cAMP-dependent protein kinase type II-alpha regulatory subunit	PRKAR2A	bovine	P00515	REARSRAST	9	S-44	GSK3	**4407.1**
Vitronectin [precursor]	VTN	human	P04004	SRRPSRATW	9	S-397	PKA	**4181.5**
Keratin, type II cytoskeletal 8	KRT8	human	P05787	FSSRSYTSG	9	S-23	/	**3799.4**
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1	PFKFB1	rat	P07953	RRRGSSIPQ	9	S-32	PKA	**3746.0**
Ras-related protein Rab-4A	RAB4A	human	P20338	RQLRSPRRT	9	S-199	p34cdc2	**3628.9**
ATP citrate synthase	ACLY	rat	P16638	TRAPSRTAS	9	S-450	MFPK	**3514.9**
Myosin IC heavy chain	MYSC	macaque	P10569	RGRSSVYSA	9	S-311	myosin	**3502.2**
Hepatocyte growth factor receptor [precursor]	MET	human	P08581	DRLVSARSV	9	S-985	PKC	**2892.5**
Acetylcholine receptor protein, delta subunit [precursor]	ACHD	torpedo californica	P02718	RRSSSVGYI	9	S-383	PKA	**2838.3**
glycogen (starch) synthase	GYS1	rabbit	P13834	PLSRTLSVS	9	S-7	PKC/CAMII	**2791.9**
Lysozyme C [precursor]	LYC	chicken	P00698	RGYSLG	6	S-42	PKA	**2776.0**
Interleukin-2 receptor alpha chain [precursor]	IL2RA	human	P01589	RQRKSRRTI	9	S-268	PKC	**2594.4**
Troponin I, cardiac muscle	TNNI3	rabbit	P02646	GRALSTRAQ	9	S-72	PhK	**2521.8**
Troponin I, cardiac muscle	TNNI3	rabbit	P02646	RRRS	4	S-20 (V17R)	PKA	**2010.5**
Glycogen phosphorylase	GPH1	yeast	P06738	TRRLTGFLP	9	T-30	/	**2475.4**
Desmin	DES	chicken	P02542	GSRGSGSSV	9	S-35	PKA	**2451.3**
Desmin	DES	chicken	P02542	QRVSSYRRT	9	S-12	PKC	**2234.4**
Ryanodine receptor 2	RYR2	rabbit	P30957	TRRISQTSQ	9	S-2809	CaM-II	**2394.6**
Lamin-A	LMNA	human	P02545	RGRASSHSS	9	S-403	PKC	**2303.7**
Lamin-A	LMNA	human	P02545	TVTRSYRSV	9	S-625	PKC	**2030.6**
Inositol 1,4,5-trisphosphate receptor type 1	ITPR1	rat	P29994	GRRESLTSF	9	S-1755	PKA	**2290.7**
Vimentin	VIM	mouse	P20152	STSRSLYSS	9	S-50	PKA	**2181.2**
Vimentin	VIM	mouse	P20152	SRPSSNRSY	9	S-25	autophosphorylation	**2147.0**
Lamin-B1	LMNB1	human	P20700	RASSSRSVR	9	S-405	PKC	**2084.2**
Fibrinogen alpha chain [precursor]	FGA	human	P02671	TTRRSASKT	9	S-460	PKA	**2036.1**
Parathyroid hormone/parathyroid hormone-related peptide receptor [precursor]	PTH1R	Didelphis marsupialis virginiana	P25107	ARSGSSTYS	9	S-485	PKA	**2029.3**
Protein phosphatase 1 regulatory subunit 3A	PPP1R3A	rabbit	Q00756	SPQPSRRGS	9	S-44	GSK3	**2004.9**

Table S1. Rank order of the 75 most strongly phosphorylated PASKIN kinase targets on the peptide microarray.

Target Sequence (Gene Symbol)	Biotinylated Peptide	Swissprot	Described Phosphorylation sites	Kinase	References
GYS	631-AQGYRYPRPASVPPSPSLSR-650	P13807	S641	PASKIN, GSK3	[1,2]
			S645	GSK3	[2]
			S649	GSK3	[2]
PDX1	141-AEPEENKRTRTAYTRAQLLE-160	P52945	T152	PASKIN	[3]
PIAS1	500-HQASPVSRTPSLPAVDTSYI-519	O75925	S503	unknown	[4,5]
			S510	unknown	[5]
RAB11BP	427-KKPESRRSSLLSLMTGKKDV-446	Q6WKZ4	S435	unknown	[4,6]
FXN	48-ATCTPRRASSNQRGLNQIWN-67	Q16595	none		
HERG	276-CASVRRASSADDIEAMRAGV-295	Q12809	S283	PKA	[7]
CREB1	120-DSQKRREILSRRPSYRKILN-139	P16220	S121	several	[8]
			S129	several	[8]
			S133	several	[8]
NFATC4	280-ASPALSRRGSLGEEGSEPPP-299	Q14934	S281	RSK2	[9]
			S285	RSK2	[9]
			S289	RSK2	[9]
4E-T	343-NPSRSGSRSSSLGSTPHEEL-362	Q9NRA8	S345	unknown	[4]
RPS6	226-EQIAKRRRLSSLRASTSKSE-245	P62753	S235	S6K/RSK	[10,11]
			S236	S6K/RSK	[10,11]
			S240	S6K	[10]
			S244	S6K	[10]
PHKB	17-ERRARTKRSGSVYEPLKSIN-36	Q93100	S25	unknown	[12]
			S27	unknown	[4,12]
PFKFB	26-RLQRRRGSSIPQFTNSPTMV-45	P16118	S33	PKA, PKG	[13]
			S34	PKA, PKG	[13]

Table S2. Sequences of the eleven biotinylated peptides tested for phosphorylation by recombinant PASKIN. Control peptides were derived from glycogen synthase(GYS) and pancreatic and duodenal homeobox (PDX)1 which have been reported previously to be phosphorylated by PASKIN. The other sequences tested were derived from the peptide microarray (underlined) and extended to 20 amino acids as indicated. Because some of the short sequences of the peptide microarray form consensus motifs, they can be found in multiple proteins. Therefore, some of the designations of the peptide microarray differ from the 20-mer biotinylated peptides. Abbreviations: GYS, glycogen synthase; PDX1, pancreatic and duodenal homeobox 1; PIAS1, protein inhibitor of activated STAT 1; RAB11BP, Rab11-binding protein; FXN, frataxin; HERG, human ether-a-go-go related gene; CREB1, cAMP response element-binding protein 1; NFATC4, nuclear factor of activated T-cells c4; 4E-T, eIF4E-transporter; RPS6, 40S ribosomal protein S6; PHKB, phosphorylase kinase β; PFKFB, 6-phosphofructokinase/fructose-2,6-bisphosphatase; MAPT, microtubule-associated protein tau; CACNA1S, calcium channel, voltage dependent, L type, alpha 1S subunit; PKLR, pyruvate kinase, liver and red blood cell; GSK3, glycogen synthase kinase 3; PKA, protein kinase A; S6K, p70 S6 kinase; RSK, p90 ribosomal S6 kinase; PKG, protein kinase G.

References

1 Wilson WA, Skurat AV, Probst B, de Paoli-Roach A, Roach PJ & Rutter J (2005) Control of mammalian glycogen synthase by PAS kinase. *Proc Natl Acad Sci U S A* **102**, 16596-16601.

2 Fiol CJ, Mahrenholz AM, Wang Y, Roeske RW & Roach PJ (1987) Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. *J Biol Chem* **262**, 14042-14048.

3 An R, da Silva Xavier G, Hao HX, Semplici F, Rutter J & Rutter GA (2006) Regulation by Per-Arnt-Sim (PAS) kinase of pancreatic duodenal homeobox-1 nuclear import in pancreatic beta-cells. *Biochem Soc Trans* **34**, 791-793.

4 Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalarski CE, Elledge SJ & Gygi SP (2008) A quantitative atlas of mitotic phosphorylation. *Proc Natl Acad Sci U S A* **105**, 10762-10767.

5 Mayya V, Lundgren DH, Hwang SI, Rezaul K, Wu L, Eng JK, Rodionov V & Han DK (2009) Quantitative phosphoproteomic analysis of T cell receptor signaling reveals system-wide modulation of protein-protein interactions. *Sci Signal* **2**, ra46.

6 Daub H, Olsen JV, Bairlein M, Gnad F, Oppermann FS, Korner R, Greff Z, Keri G, Stemmann O & Mann M (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Mol Cell* **31**, 438-448.

7 Cui J, Melman Y, Palma E, Fishman GI & McDonald TV (2000) Cyclic AMP regulates the HERG K(+) channel by dual pathways. *Curr Biol* **10**, 671-674.

8 Johannessen M & Moens U (2007) Multisite phosphorylation of the cAMP response element-binding protein (CREB) by a diversity of protein kinases. *Front Biosci* **12**, 1814-1832.

9 Cho YY, Yao K, Bode AM, Bergen HR, 3rd, Madden BJ, Oh SM, Ermakova S, Kang BS, Choi HS, Shim JH & Dong Z (2007) RSK2 mediates muscle cell differentiation through regulation of NFAT3. *J Biol Chem* **282**, 8380-8392.

10 Jeno P, Ballou LM, Novak-Hofer I & Thomas G (1988) Identification and characterization of a mitogen-activated S6 kinase. *Proc Natl Acad Sci U S A* **85**, 406-410.

11 Erikson E & Maller JL (1985) A protein kinase from Xenopus eggs specific for ribosomal protein S6. *Proc Natl Acad Sci U S A* **82**, 742-746.

12 Oppermann FS, Gnad F, Olsen JV, Hornberger R, Greff Z, Keri G, Mann M & Daub H (2009) Large-scale proteomics analysis of the human kinome. *Mol Cell Proteomics* **8**, 1751-1764.

13 Glass DB, el-Maghrabi MR & Pilkis SJ (1986) Synthetic peptides corresponding to the site phosphorylated in 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as substrates of cyclic nucleotide-dependent protein kinases. *J Biol Chem* **261**, 2987-2993.

8 MANUSCRIPT IV (UNPUBLISHED)

Paskin$^{-/-}$ mice are protected from detrimental effects of obesity

Philipp Schläfli[1], Emanuela Borter[1], Peter Y. Wielinga[2], Jacquelien J. Hillebrand[3], Patrick Spielmann[1], Christian Löwenstein[2], Bettina Alder[2], Irene Tobler[4], Thomas A. Lutz[2], and Roland H. Wenger[1]

[1]Institute of Physiology and Center for Integrative Human Physiology, University of Zürich, CH-8057 Zürich, Switzerland

[2]Institute of Veterinary Physiology and Zurich Center for Integrative Human Physiology, Vetsuisse Faculty University of Zürich, CH-8057 Zürich, Switzerland

[3]Institute of Animal Sciences, ETH Zürich, CH-8603 Schwerzenbach, Switzerland[2]

[4]Institute of Pharmacology and Toxicology and Neuroscience Center Zürich, University of Zürich, CH-8057 Zürich, Switzerland

ABSTRACT

PASKIN is a PAS domain containing AMP-related serine/threonine sensory kinase which is ubiquitously expressed at low levels with highest expression in testis. We previously generated PASKIN knock-out mice that did not display an apparent phenotype. However, a function for PASKIN in the regulation of insulin and glucagon expression and secretion has been reported. Glucose stimulated insulin expression in pancreatic β-cells relies on the activity of the transcription factor pancreatic and duodenal homeobox protein 1 (PDX-1). It has been shown that PASKIN modulates the both expression and activity of PDX-1. In yeast, a PASKIN phenotype only appears under high temperature stress conditions with galactose as only energy source, where the yeast orthologue Psk phosphorylates target proteins involved in energy metabolism and protein translation. One of the targets is yeast glycogen synthase, of which also its mammalian orthologue can be phosphorylated by PASKIN leading to a breakdown of intracellular glycogen. We speculate that PASKIN might sense the concentration of an unknown metabolic ligand and signal as a sensor of

metabolic homeostasis. Herein we show that upon feeding a 45% high fat diet, *Paskin*$^{-/-}$ mice are partially protected from obesity and insulin resistance. However, feeding of a 60% high fat diet did not increase the phenotype, in the contrary, any differences in body weight gain disappeared. Furthermore, we could not find a hypermetabolic phenotype in indirect calorimetry and body temperature measurements. We demonstrate that energy homeostasis mediated by PASKIN on cellular level is independent of AMPK but might involve thyroid hormones which negatively influence PASKIN kinase activity *in vitro* and therefore might be a repressive metabolic ligand of PASKIN.

INTRODUCTION

PAS (Per-Arnt-Sim) proteins are known from all three kingdoms of life where they represent protein sensors involved in the perception of light intensity, oxygen partial pressure, redox potentials, voltage and certain ligands (*1-2*). Mammalian PAS domains can also serve as heterodimerisation interfaces of transcription factors involved in xenobiotic response, adaptation to hypoxia and circadian rhythm generation (3-6). By database searches using the PAS sequence as a bait, we and others identified a mammalian PAS protein termed PASKIN (7) or PASK (8). PASKIN contains two PAS domains showing a high sequence similarity to the oxygen sensor protein FixL of *Rhizobium* species, and a serine/threonine kinase domain related to the AMP-kinases. According to the current model, the PAS domain of PASKIN represses the kinase activity in *cis*. Upon binding of a ligand to the PAS domain, the kinase domain is de-repressed, therefore activated and can autophosphorylate (9). Synthetic ligands have been identified that show a structural similarity to dioxin, a molecule known to bind the PAS domain of the aryl hydrocarbon receptor (9). However, an endogenous ligand for PASKIN activation has not been identified so far.

To elucidate the function of PASKIN, our lab previously generated PASKIN deficient knock-out mice (10). We found that PASKIN mRNA expression was remarkably higher in testis than in any other tissue (11). Immunohistochemistry showed that PASKIN is upregulated during the post-meiotic stages of spermatogenesis and concentrates at the mid-piece of the sperm tail (12). Functionally, however, neither male fertility nor sperm production and motility was affected in PASKIN knock-out mice (10).

In yeast, a double knockout of the PASKIN homologs PSK1 and PSK2 displayed decreased growth at elevated temperature (39°C) and galactose supply (13). PSK1 and PSK2 phosphorylate three translation factors (Caf20, Tif11 and Sro9) and two enzymes involved in glycogen and trehalose synthesis (Ugp1, Gsy2), thus controlling translation and sugar flux (13). It has been shown that mammalian glycogen synthase interacts with the midregion of human PASKIN and can be phosphorylated at Ser-640 (14). However, mammalian PASKIN has mainly been involved in insulin signaling. In pancreatic β-cells PASKIN mRNA is upregulated upon glucose stimulation (*15-16*). Several findings involve PASKIN in upregulation of PDX-1 mRNA (*15-16*), a major transcription factor of the insulin gene, and it has been shown that PASKIN kinase function might phosphorylate PDX-1, leading to translocation of the transcription factor into the nucleus (17). However, we have challenged the role for PASKIN in glucose stimulated insulin gene expression as in our hands Paskin mRNA levels were not increased upon glucose stimulation (11).

Using our *Paskin*$^{-/-}$ mouse model it has been found, that male *Paskin*$^{-/-}$ mice were partially protected from high fat diet induced obesity and showed better glucose tolerance (18). This

observation together with increased O_2 consumption and CO_2 production led to the hypothesis that *Paskin*$^{-/-}$ male mice were hypermetabolic. Futhermore, it has been shown that ATP synthesis rate was increased in *Paskin*$^{-/-}$ soleus muscle and a knockdown of Paskin in L6 myoblasts resulted in increased glucose and palmitate oxidation (18). It is well acknowledged that the main cellular metabolic sensor is represented by AMP activated protein kinase (AMPK), a well-conserved serine/threonine kinase (19). AMPK is formed by a catalytic subunit (α) and two regulatory subunits (β and γ) and is activated allosterically by an increased intracellular AMP/ATP ratio and by phosphorylation of the threonine 172 residue located in the activation loop of the α subunit (20). Once activated, AMPK leads to a concomitant inhibition of energy-consuming biosynthetic pathways and activation of ATP-producing catabolic pathways.

Herein we show that PASKIN is to some extent a direct counterplayer of AMPK signaling pathway. By changing the diet to 45% fat by calories, *Paskin*$^{-/-}$ mice resisted to fat accumulation and glucose intolerance. However, elevating the fat content to 60% fat by calories did not show any further increase of the phenotype, in the contrary, the phenotypic differences were lost. To find potential cellular explanations, we particularly focused on AMPK signaling and growth of mouse embryonic fibroblasts and thyroid hormone as potential ligand of the PAS domain of PASKIN.

METHODS

Animals

Paskin$^{-/-}$ mice (10) were backcrossed into C57BL/6J (The Jackson Laboratory) for 10 generations. C57BL/6J control animals were raised in the same room under the same conditions. Mice were maintained on a normal chow diet or a high fat diet beginning at 12 weeks of age (Research Diets, D12451 with 45% fat by calories, D12492 with 60% fat by calories). In each experiment, age-matched wild type were used as controls for *Paskin*$^{-/-}$ mice. All animal handling followed the guidelines for the use and care of laboratory animals of the Bundesamt für Veterinärwesen and was approved by the Kantonales Veterinäramt Zürich (Nr. 192/2003 and 8/2008).

Immunoblotting

Total protein extracts of cultured cells were prepared using cell lysis buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 400 mM NaCl, 0.1% NP-40, protease inhibitor cocktail (Roche)) and centrifuged (5 min, 15´000 rpm, 4°C). Proteins from tissues were prepared as described previously (21). Concentrations of supernatants were determined according to Bradford (22).

Proteins were seperated by SDS-PAGE and transferred onto nitrocellulose membranes (Amersham). The following primary antibodies were used: rabbit monoclonal antibody (mAb) anti-AMPKα (Cell signaling) and rabbit mAb anti-phospho-AMPKα (T172) (Cell signaling), rabbit polyclonal anti-β-actin (Sigma); and secondary polyclonal goat anti-mouse or anti-rabbit antibodies coupled to horseradish peroxidase (Pierce) Chemiluminescence detection was performed with the Supersignal West Dura kit (Pierce) and recorded with a CCD camera (FluorChem8900, AlphaInnotech, Switzerland) or by exposure to X-ray films (Fuji).

Motor activity recordings and analysis

Mice were housed individually in sound-proof and light-tight environment with a light/dark cycle of 12 h each in cages equipped with a running wheel or infrared sensors. Wheel revolutions were monitored in 1 minute intervals continuously using the Chronobiology Kit (Stanford Software Systems) as described previously (23). The experiment was conducted for the indicated number of animals at the age of 11-13 weeks for 10 consecutive days.

Glucose / insulin tolerance test

Animals tested were 9 months of age and were fed a HFD for 6 months. For glucose tolerance tests (GTT), the animals were fasted for 16 h before injection of 20% glucose in 0.9% NaCl (2 g glucose/kg body weight) i.p. At the indicated times, tail vein blood was sampled for electrochemical glucose determination (Accu-Chek Aviva, Roche Diagnostics). For insulin tolerance tests (ITT), human recombinant insulin (0.75 U/kg body weight) was injected intraperitoneally to fasted (16 h) mice and blood glucose levels were determined at the indicated times.

Indirect calorimetry

At 14 weeks of age, mice were fed a 60% high fat diet. For assessment of body temperature, mice were implanted a transmitter (PhysioTel PA-C10, Data Science International, St. Paul, MN) into the peritoneal cavity under isoflurane anesthesia at 18 weeks of age. This transmitter allowed telemetric assessment of body temperature and physical activity. Body temperature and physical activity were simultaneously monitored by the DataScience ART4.0 telemetry system (DataScience International, St. Paul, MN). Indirect calorimetry measurements were performed at 28 weeks of age in an open circuit calorimetry system (AccuScan Inc., Columbus, OH). Room air was passed through each cage with a flow rate controlled at approximately 2 l/min. Every 2 min, air was sampled during 20 s for each individual cage and analyzed for $V(O_2)$ and $V(CO_2)$ (ml/kg/min). All data were analyzed with

AccuScan Integra ME software. Energy expenditure was calculated according to Weir (24) using the following equation: total energy expenditure (kcal/h) = 3.9 × V(O_2) ml/h + 1.1 × V(CO_2) ml/h. The respiratory quotient (RQ) is defined as the quotient between CO_2 production and O_2 consumption.

Metabolic blood analysis

Paskin$^{-/-}$ and *Paskin*$^{+/+}$ mice were 11 months old and one group had been fed a 45% HFD for the last 8 months. Animals were fasted for 6 hours before anaesthesia (200 mg/kg body weight Ketamine, 10 mg/kg body weight Xylazine). Up to 1 ml blood was removed from the portal vein into a heparinised syringe with a 25 Gauge needle. After centrifugation (5 min, 8000 g, 25°C) the plasma was stored at -80°C. Measurements of indicated analytes were performed by the institute for clinical chemistry (IKC, USZ Zürich, Switzerland). For the measurement of total T3 and total T4 thyroid hormones, mice were anesthesized and blood was removed by heart puncture. Plasma was analysed by Unilabs Dr. Weber (St. Gallen, Switzerland).

Intracellular ATP measurements and MEF growth analysis

ATP measurements were performed in 96-well format. 5000 cells were seeded per well and grown in Dulbecco's modified eagles medium with 4.5 g glucose per liter. 16 hours post seeding, cells were washed three times with PBS and supplemented with media of different glucose concentrations with or without fetal calf serum for 2 hours, as indicated. Thereafter, ATP concentration was measured by a luminescence based assay using CellTiter-GloR reagent (Promega). Measurements were performed by mixing the cells with 25 µl of DMEM without FCS and 25 µl of CellTiter-GloR reagent. After 10 minutes of passive lysis whith vertical shaking, luminescence of lysates was measured on a luminometer (Centro LB 960, Berthold Technologies), and quantified versus an ATP standard dilution series ranging from 1 nM to 10 µM. For normalisation, protein concentration of entire wells was determined by BCA assay (Pierce). Growth curves and cell size were analysed using a cell counter (ViCell, Beckman Coulter).

Kinase assay

Recombinant proteins were purified as described previously (12). Briefly, full-length PASKIN was expressed in Sf9 cells using the Bac-to-Bac Baculovirus expression system (Invitrogen). Recombinant His_6-PASKIN was purified by FPLC (BioLogic DuoFlow, Biorad) using a HiTrap Chelating HP column (GE Healthcare). The kinase activity of purified recombinant PASKIN was verified by auto-phosphorylation assays. His_6-PASKIN was

incubated in kinase buffer (25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) for 10 minutes in presence of 3 μCi [γ-^{33}P]-ATP (Hartmann Analytic). The individual reactions contained thyroid hormones (Sigma) in a 10-fold serial dilution ranging from 100 μM to 1 nM (dissolved in 50 mM NaOH added 1:10 to each reaction) including a solvent control. Reactions were stopped with 1% SDS, separated by SDS-PAGE and analyzed by phosphorimaging of the dried gels (Molecular Imager FX, Bio-Rad) using QuantityOne software (Bio-Rad).

Computed tomography

Mice fed a 60% high fat diet were analysed for body fat content and liver steatosis *post mortem* using a La Theta LCT-100 (Aloka) tomograph as previously described (25). Briefly, X-ray source voltage was set to 50 kV with a constant current of 1 mA. Mice were measured in a 48 mm holder at 1 mm pitch size in high speed settings. Mice tails were excluded from measurements, as they contained negligible amounts of fat. The experiment was performed using n=6 *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice, respectively. Using Aloka software, subcutaneous and intraabdominal fat were separated by the abdominal wall for estimation. Liver fat content was determined from two sequential slices where liver, spleen and adipose tissue were simultaneously present. In humans, attenuation of liver is at least 4 Hounsfield units greater than for spleen, whereas in individuals with hepatic steatosis, liver attenuation is smaller than spleen attenuation (26). Attenuations were determined by averaging the Hounsfield units of two adjacent slices and liver/fat and liver/spleen ratios as well as liver-spleen differences were calculated.

RESULTS

Body weight of *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice

Body weight of *Paskin*$^{-/-}$ mice was analysed on a daily base from day 6 on and after weaning on a weekly base until the age of three months. The graphs in Fig. 1A represent the mean weight of the animals at an age of 65 days, when a small but significant elevation of body weight could be detected in male but not female *Paskin*$^{-/-}$ mice.

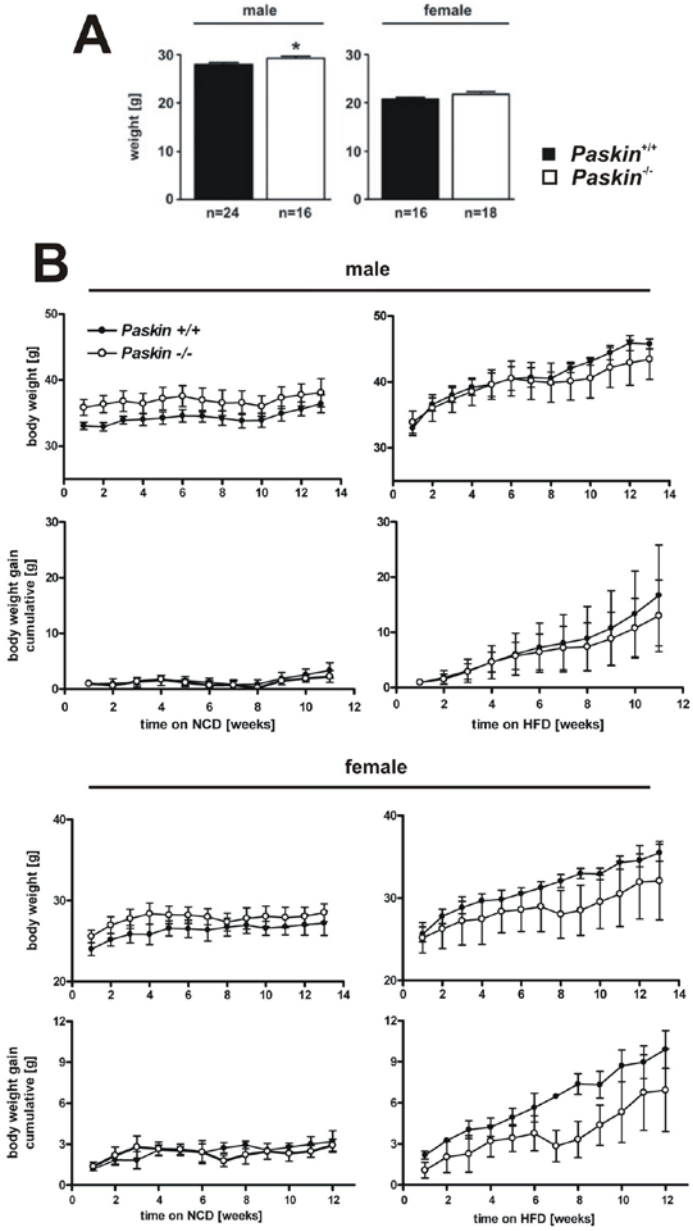


Fig. 1. Body weight of *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice under chow diet and high fat diet. (A) *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice under normal chow diet. The body weight at the age of 65 days is shown for the indicated number of animals (mean ± SEM, Student's t-test, * $p<0.05$). (B) The left panel shows body weight and body weight gain for male mice under chow diet and 45% high fat diet (n=8 mice per group, high fat diet started at the age of 14 weeks). The right panel shows female mice, accordingly. Despite similar food intake (data not shown), *Paskin*$^{-/-}$ animals gain less body weight under a 45% high fat diet. Data are represented as mean ± SEM.

To challenge metabolic homeostasis, *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice were fed a diet consisting of 45% fat by calories. At the age of 14 weeks, 16 *Paskin*$^{-/-}$ mice (8 females and 8 males) were fed either a high fat diet (HFD) or normal chow diet (NCD, 11.7% fat by calories). Analogously, 16 *Paskin*$^{+/+}$ control animals were included. The left panels of Fig. 1B represent the body weight and the cumulative body weight gain under NCD for male and female mice, respectively, while on the right panels the same evaluation is illustrated under HFD conditions. Male *Paskin*$^{-/-}$ mice showed the expected significant increase in body weight on NCD but no difference in cumulative body weight gain. Under HFD, however, *Paskin*$^{-/-}$ mice showed a lower body weight after 8 weeks on HFD compared to the body weight of *Paskin*$^{+/+}$ mice as a result from decreased body weight gain under HFD. Similarly, female *Paskin*$^{-/-}$ mice were heavier on NCD but gain less body weight under HFD. Thus, the protective effect on development of obesity for male *Paskin*$^{-/-}$ animals on HFD, as published before (18), could be reproduced in our hands. In our feeding experiment this result holds true for female *Paskin*$^{-/-}$ mice as well. This encouraged us to investigate the metabolic phenotype in more detail.

Intraperitoneal glucose tolerance tests with HFD fed mice

Feeding of a HFD induces obesity and is a model the metabolic syndrome (27). The maintainance of stable basal blood glucose levels is ensured by the peptide hormones insulin and glucagon. Type 2 diabetic patients usually show peripheral insulin resistance as a consequence of hyperglycemia. Intraperitoneal glucose tolerance and insulin tolerance tests (IPGTT/IPITT) are standard methods to phenotype strains with impaired glucose homeostasis. We have previously shown that under NCD the ability of glucose clearance after injected glucose is identical between *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice (11). However, it has been published that male *Paskin*$^{-/-}$ mice appeared to be partially protected from insulin resistance when fed a HFD (18). As expected, we did not observe any difference in IPGTT for NCD fed mice (Fig. 2A, left panel) and only HFD fed male but not female *Paskin*$^{-/-}$ mice showed a partial protection from insulin resistance. Therefore we assume that HFD-feeding causes no defect in glucose tolerance in *Paskin*$^{-/-}$ males. According to published literature, this phenotype does not occur in *Paskin*$^{-/-}$ females on HFD, confirming our results (18).

Insulin tolerance tests were performed with the same animals one week later. In this experiment, especially male animals hardly lowered their blood glucose levels independent of their genotype. However, the blood glucose levels of HFD fed *Paskin*$^{-/-}$ males were generally lower for the whole duration of the insulin tolerance test. Female mice showed a drop of 40 mg/dl, however, the fasted blood glucose levels were already low in NCD animals (80 mg/dl). This result is contradictory to published observations on randomly HFD fed animals, where increased insulin sensitivity in male *Paskin*$^{-/-}$ mice was observed (18).

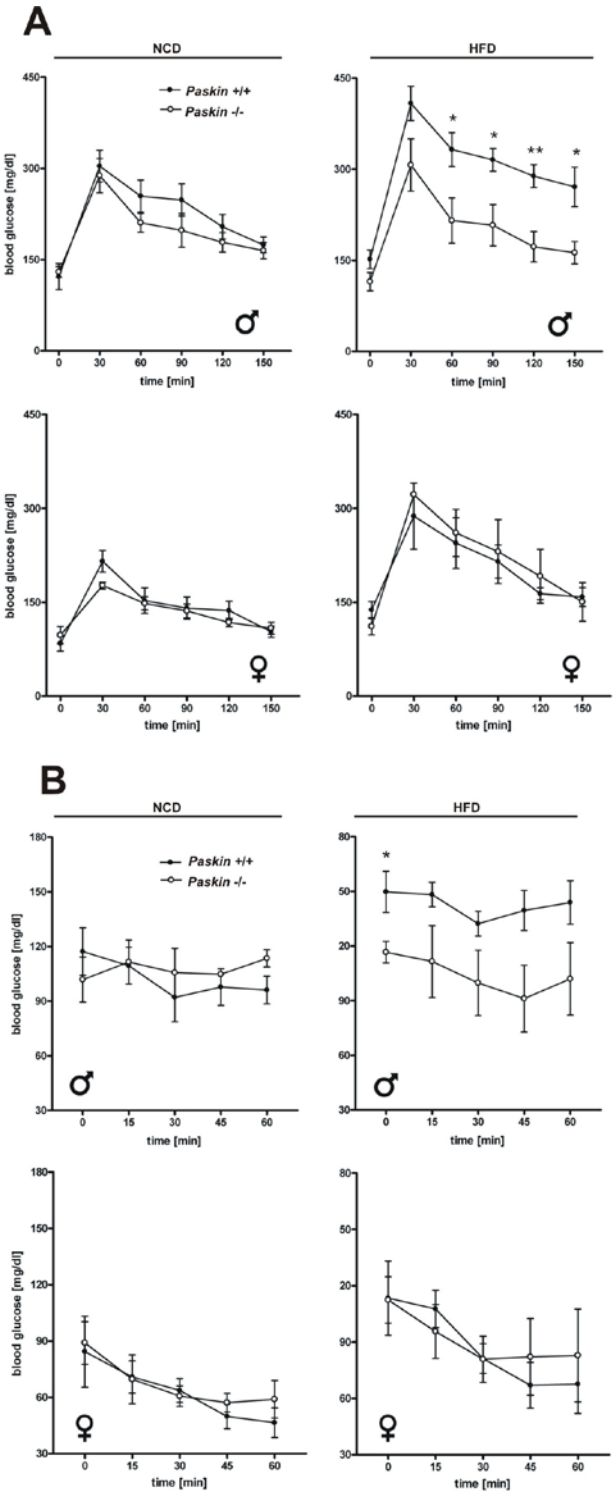


Fig. 2. Glucose tolerance test and insulin tolerance test for *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice under chow diet and high fat diet. (A) Glucose tolerance test for *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice under normal chow diet and high fat diet. Fasted mice were administered 2 g/kg body weight of glucose intraperitoneally. Each group consisted of three to four animals at the age of 9 months. Only male mice fed a 45% high fat diet display an effect of partial protection of an insulin resistance for the *Paskin*$^{-/-}$ genotype. (B) Intraperitoneal insulin tolerance test of the same animals as in (A). Fasted mice were administered 0.75 U/kg body weight of human insulin. No difference could be found. Although *Paskin*$^{-/-}$ male mice generally had lower glucose levels than *Paskin*$^{+/+}$ males, the animals did not raise their blood glucose levels prior to the insulin tolerance test and did not respond to administered insulin. Data are represented as mean ± SEM (Student's t-test, * $p<0.05$, ** $p<0.01$).

Indirect calorimetry measurements with *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice

Since we could confirm that at least male *Paskin*$^{-/-}$ mice are partially protected from high fat diet induced obesity and might be hypermetabolic, a second round of feeding experiments was started using a diet consisting of 60% of fat by calories instead of 45%, with the goal of increasing potential phenotypic effects on body weight. The body weight of 7 *Paskin*$^{-/-}$ and 7 *Paskin*$^{+/+}$ male mice on 60% fat HFD was monitored for 16 weeks. Fig. 3A, however, shows that instead of increasing the protective effect on obesity (as shown in Fig. 1B), *Paskin*$^{-/-}$ males did not differ from control animals following an increase in dietary fat content from 45% to 60%.

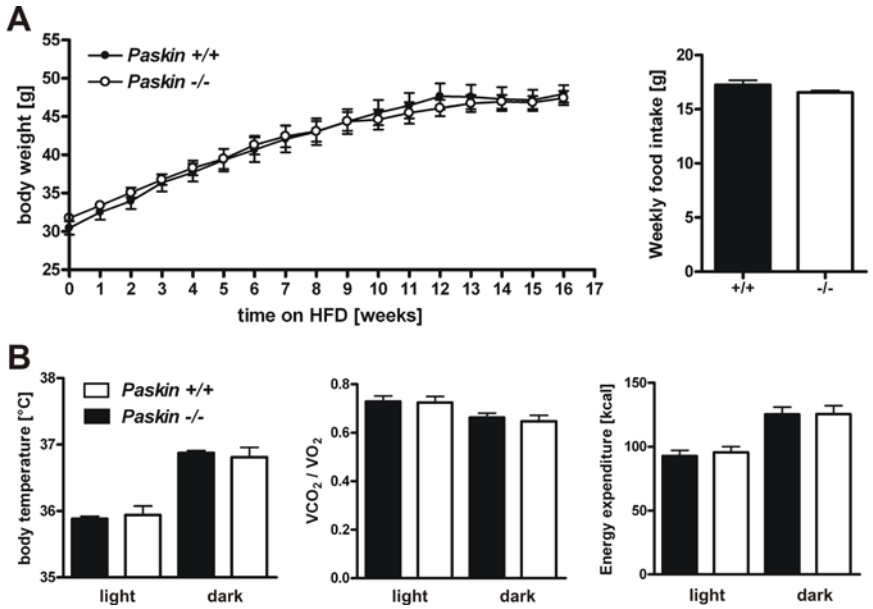


Fig. 3. Feeding of a 60% fat by calories high fat diet and indirect calorimetry for *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ male mice. (A) Growth curve for *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ male mice under a high fat diet of 60% of fat by calories. *Paskin*$^{-/-}$ males were slightly heavier at the age of 14 weeks, when the HFD feeding was initiated. The slight difference in body weight gain can be explained by a slightly lower food intake for *Paskin*$^{-/-}$ mice. Instead of increasing the effect seen for a 45% HFD (Fig. 1), the 60% HFD did not show any significant difference between *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ male mice (n=7 mice per group). (B) To see a potential difference in energy expenditure, we performed indirect calorimetry experiments for male mice fed a 60% HFD including the continuous recording of body temperature by a sensor implanted intraperitoneally. While the animals were eating equal amounts of food and showed stable body weight, we expected differences in body temperature or energy expenditure. Analysis was split into 12 h light and 12 h dark phase but did not show differences in any of the parameters measured for an n = 6 animals. Data are represented as mean ± SEM.

Obesity occurs from an imbalance between energy intake (food intake) and energy expenditure (basal metabolism and physical activity) (28). Therefore, we were interested, whether the hypermetabolic phenotype was manifested in indirect calorimetry hypothesizing that despite similar body weight, energy expenditure was impaired. Additionally, we intraperitoneally implanted sensors that allowed a continuous measurement of body temperature. We conducted this experiment on male *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice on 60% high

fat diet with n=6 animals each group. The mice were adapted to the metabolic cages for 5 days before measurement. The analysis was split into a 12 hour light phase and a 12 hour dark phase. As expected, animals were more active during dark phase showing increased energy expenditure, respiratory quotient and body temperature (Fig. 3B). However, we could not confirm a *Paskin*$^{-/-}$ specific hypermetabolic phenotype. During the measurement, both groups consumed equal amounts of food and displayed equal body weight gain (data not shown) while not differing in any of the calorimetric parameters measured.

Running Wheel performance of *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice

Another possible parameter that might lead to differences in body weight is locomotor activity. The fact that male *Paskin*$^{-/-}$ mice under NCD are slightly heavier might be caused by a lower spontaneous activity. The extent of locomotor activity was assessed with infrared sensors as well as by monitoring running wheel activity in a normal 12h dark/12h light cycle. For running wheel activity, we included 14 *Paskin*$^{+/+}$ and 21 *Paskin*$^{-/-}$ male mice and for IR-beam breaks we tested 6 *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ males, each. The locomotor activity for each setup was recorded for 10 subsequent days. As expected, the animals were barely active during the light phase, not running more than an average of 101 meters per day and showing an IR-beam break activity 10 fold lower than during the dark phase. Therefore the run distance in the dark phase is shown. For every animal, the average distance covered in the running wheel per 12 hours dark phase was calculated (Fig 4A). However, the average running wheel distances do not differ between *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice with 11.6±0.9 and 11.9±0.5 km per night, respectively. More random and subtle movements in the cages were assessed by IR-beam break activity. Fig 4B shows the average number of IR-beam breaks per 12 h dark phase. Confirming the result from the running wheel, no difference in activity could be observed.

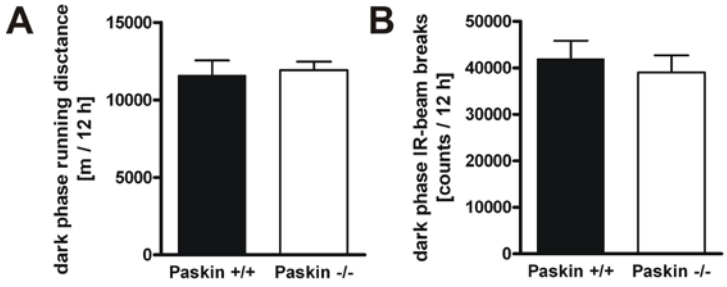


Fig. 4. Basal locomotor activity of *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ male mice. (A) Mice were kept in cages with a running wheel option with a LD cycle of 12 h. As the animals were barely active during the light phase, dark phase activity is shown as m / 12 h averaged over 10 days of experimentation. No difference between *Paskin*$^{+/+}$ (n = 14) and *Paskin*$^{-/-}$ (n = 21) male mice can be observed. (B) For n = 6 mice per group, locomotor activity was assessed by infrared beam breaks. Dark phase activity is displayed similar to panel (A). No difference between *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ male mice could be observed in this experiment. All data are represented as mean ± SEM.

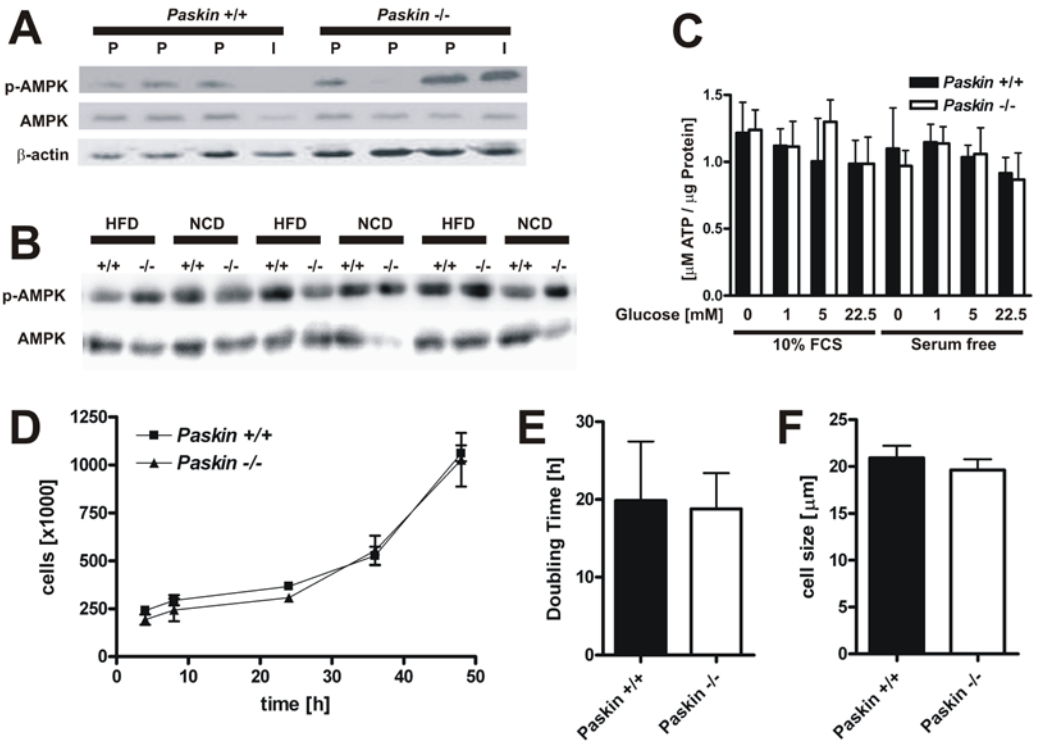


Fig. 5. Cellular energy status in *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ MEFs. (A) The cellular energy status of *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ MEFs was analysed for phosphorylation of AMP kinase in three primary isolates (P) and spontaneously immortalised (I) MEFs. Phosphorylation of AMP kinase at T172 activates the enzyme when energy status is low. A tendency to stronger phosphorylation of *Paskin*$^{-/-}$ MEFs can be observed and would support a hypermetabolic phenotype. (B) In gastrocnemius muscle extracts from mice fed either a HFD or a NCD, no difference in phospho-AMPK between *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice could be observed. (C) Intracellular levels of ATP in immortalised MEFs were assessed by a luciferase based assay. A total of 6 wells of a 96-well microtiter plate per growth condition were measured for ATP content and normalised to total protein. No differences could be observed for any of the conditions measured. (D, E) Immortalised MEFs were analysed for their growth behaviour. (D) represents a typical growth curve of cells seeded in quadruplicates. (E) depicts the doubling times of three independent growth curve experiments. (F) Cell size was analysed after detachment of cells in PBS/EDTA visually using ViCell counter. Altogether, immortalised MEFs from *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ embryos show similar growth.

AMPK expression in *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mouse embryonic fibroblasts

As the *in vivo* experiments showed a metabolic phenotype only for male *Paskin*$^{-/-}$ mice on 45% HFD, we were interested whether we could transduce a potential role for Paskin in energy homeostasis to a cellular level. Therefore, we isolated mouse embryonic fibroblasts (MEFs) from our *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice and examined whether PASKIN might have an effect on AMPK, which is the major sensor of the cellular metabolic energy status. We kept primary MEFs in culture until cells spontaneously immortalised. Immunoblot analysis of primary and immortalised MEFs showed a tendency to lower activity of AMPK in *Paskin*$^{+/+}$ MEFs, detected with a phospho-T172 AMPK antibody (Fig.5A). Quantitative real time PCR showed the presence of AMPK subunit α1 and not subunit α2 in our MEF cells. Furthermore we could confirm in both, the spontaneously immortalized and the primary MEF, that AMPK

is not regulated on the transcriptional level through PASKIN (data not shown). Analysing protein extracts of gastrocnemius muscle from mice fed either a NCD or a HFD did not confirm these findings (Fig. 5B).

To investigate the energy status of our immortalised MEFs more directly, we determined the intracellular ATP concentration on a firefly luciferase based readout. We seeded equal amounts of MEFs on a 96-well plate with DMEM and different glucose concentrations as indicated in Fig. 5C. Lysates generated from these cells could directly be used for determination of cellular ATP, since ATP consumed by firefly luciferase directly correlates with light emission (29-30). The levels of ATP were normalised to total protein content. Intracellular ATP, however does not differ between *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ MEFs. Furthermore, we performed growth curve experiments (Figs. 5D, 5E) and determined cell size (Fig. 5F). From three independent growth curve experiments, no difference in doubling time could be observed with average of 19.8 hours for *Paskin*$^{+/+}$ MEFs and 18.8 hours for *Paskin*$^{-/-}$ MEFs and similar cell size. Therefore we conclude that AMPK is not regulated by PASKIN and the cellular metabolic state does not differ in wildtype versus knock-out MEFs. It is, however, questionable whether MEFs are the appropriate model for a cellular metabolic phenotype to occur.

Blood plasma analysis of *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice

Blood from *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice either fed 45% HFD or chow diet was collected, and metabolites were analysed. As markers for lipid homeostasis, cholesterol and triglycerides were estimated. Total cholesterol blood levels are expected to be elevated under HFD (31). This held true for the *Paskin*$^{+/+}$ female mice (Fig. 7b). *Paskin*$^{-/-}$ females, however, had lower blood cholesterol which is consistent with the finding, that under 45% high fat diet their body weight was not elevated to the same extent as the *Paskin*$^{+/+}$ body weight. However, for male animals the contrary was found leading to very moderate effects when male and female values were taken together (Fig. 7b). High triglyceride blood levels arise as a consequence of secondary hyperlipemia and are associated with diabetes mellitus (32). Although not significant, triglyceride levels were slightly elevated in HFD fed *Paskin*$^{+/+}$ male animals when compared to *Paskin*$^{-/-}$ male mice. The contrary was true for female animals (Fig. 7c). Conversely, we detected increased free fatty acid levels in HFD and NCD fed *Paskin*$^{-/-}$ females versus *Paskin*$^{+/+}$ females (Fig. 7d).

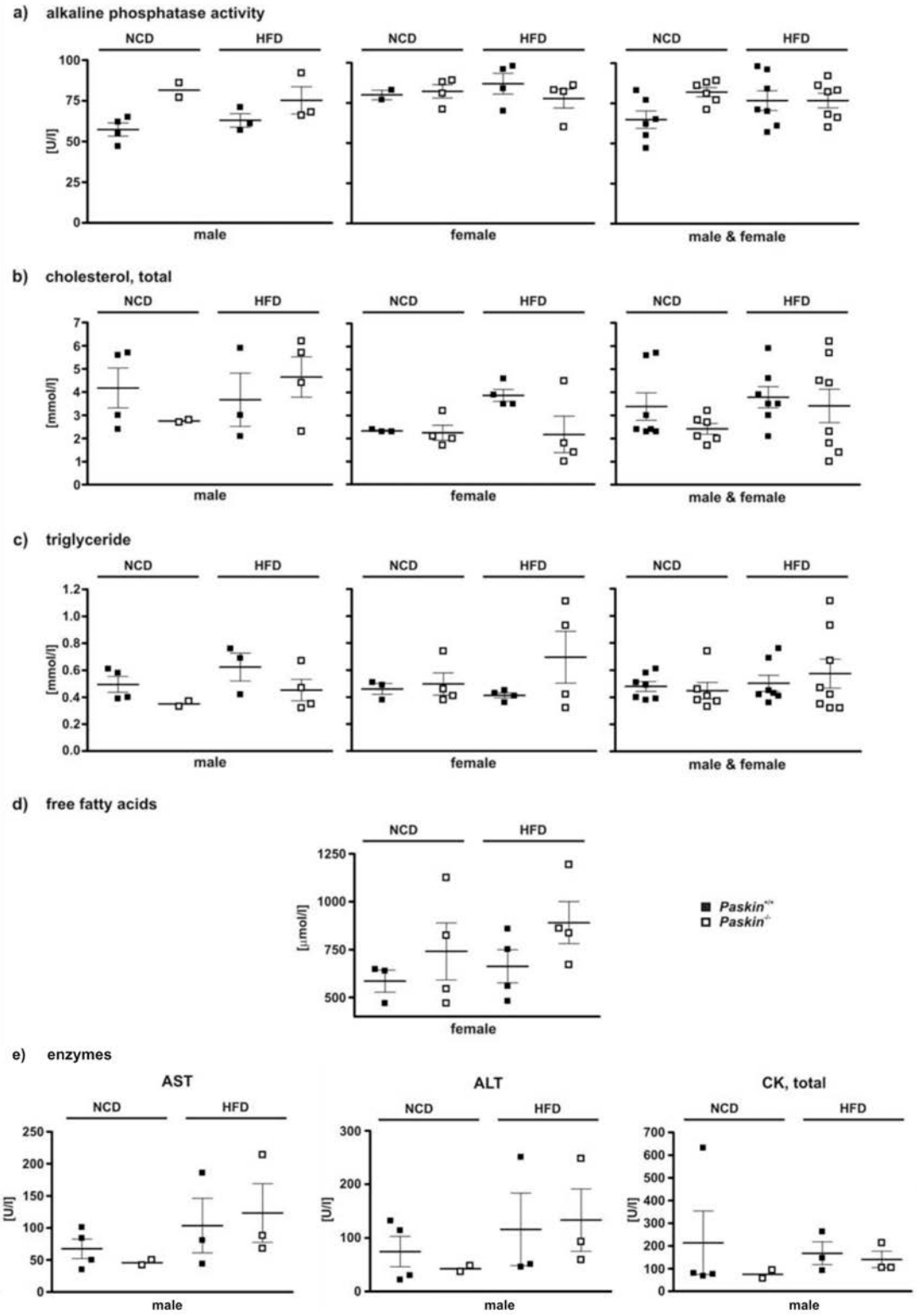


Fig. 6. Blood plasma lipid homeostasis in mice fed a chow diet or high fat diet. Mice were fasted for 6 hours prior to terminal blood sampling from portal vein. See text for detail. Data represent mean ± SEM.

Indicative enzymes for proper liver function are alanine-aminotransferase (ALT), aspartate-aminotransferase (AST) and alkaline phosphatase (ALP). It has been shown that feeding of a high fat diet induces hepatic steatosis and leads to elevated blood levels of ALT, AST and ALP (33). For ALP, our data indicate a slight increase for *Paskin*$^{-/-}$ male mice independent of the diet. For AST and ALT, one *Paskin*$^{+/+}$ and one *Paskin*$^{-/-}$ male mouse showed increased activity when fed a 45% HFD, potentially indicating diet induced steatosis, however, not differing between the two genotypes and the number of animals should be increased for valuable results. Creatine kinase (CK) is a marker enzyme for myocardial infarction and can be elevated, reflecting muscle activity. As obesity is a risk factor for myocardial infarction, this parameter is of interest, even though it has been shown that obesity itself does not alter CK activity from patient serum (34). However, no changes in blood CK levels could be observed between *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ male mice (Fig. 6e).

Conclusively, we could not find significant differences in the blood parameter analysis. However, we observed several trends, such as slightly decreased triglyceride levels in *Paskin*$^{-/-}$ male animals and elevated levels of free fatty acids in female *Paskin*$^{-/-}$ mice. This might reflect a better breakdown of triglycerides in the adipose tissue of *Paskin*$^{-/-}$ mice. The differences observed in cholesterol levels were contradictory and when both genders were analysed together, the levels were similar. Further experiments with an increased number of animals are required to get additional hints of a PASKIN function with respect to lipid metabolism. Regarding liver function, however, we did not observe any difference at all between the two groups.

Thyroid hormones enhance PASKIN autophosphorylation *in vitro*

A model for PASKIN enzyme function proposes auto-inhibition of the kinase function of PASKIN *in cis* by its PAS domain. As a potential sensory domain, a yet unknown small organic ligand is suggested to bind the PAS domain thereby inducing changes in conformation leading to de-repression of the kinase domain (9). Rutter *et al.* have screened a compound library and found that the strongest ligands binding the PAS domain consisted of two aromatic rings (9). Therefore we were interested, whether PASKIN activity monitored by autophosphorylation is influenced by the presence of the thyroid hormones thyroxine (T4) or triiodothyronine (T3), two hormones resembling the before mentioned synthetic compounds. As a control, the biosynthetic precursor amino acid L-tyrosine was used. We could observe a concentration dependent response of PASKIN autophosphorylation to both T3 and T4 with inhibtion at high concentrations and activation at low concentrations of the hormones (Fig. 7A). However, the concentrations in the *in vitro* assay are in the micromolar to nanomolar range and do not reflect the low intracellular concentrations that range in low picomolar

concentrations (35). Thyroid hormones are important regulators of cellular metabolic processes by increasing basal metabolic rate, increasing both breakdown and synthesis of cellular energy storage and protein thereby generating heat (36). We therefore wondered, whether we could observe any difference of thyroid hormones in blood plasma of *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice. As thyroid hormones circulating in the blood stream are bound to thyroid binding globulin (TBG) and thereby inactive, our measurements of total thyroid hormones rather reflect the synthesis of thyroid hormones that might largely differ from the amount of available and active hormones. As depicted in Fig. 7B, no difference in total thyroid hormones T3 and T4 was detected between *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ plasma.

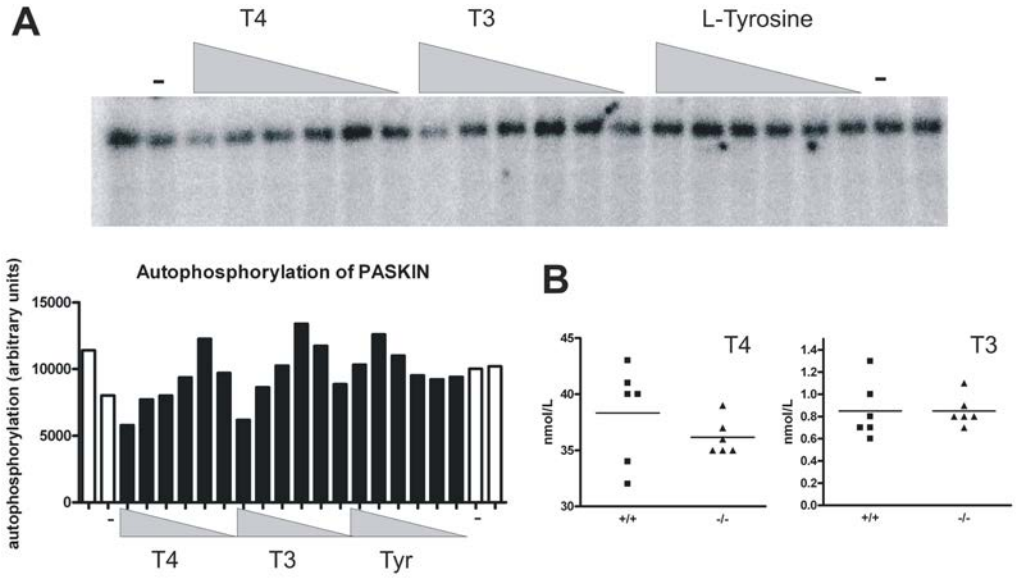


Fig. 7. Inhibition of PASKIN autophosphorylation by Thyroid hormones. (A) Recombinant PASKIN protein was allowed to autophosphorylate *in vitro* in presence of thyroid hormones T4, T3 and L-tyrosine as a control. Concentrations of thyroid hormones and L-tyrosine ranged from 100 μM to 1 nM in 10-fold dilutions. Compared to negative solvent controls (-/open bars), PASKIN autophosphorylation is inhibited by approximately 50% with a concentration of 100 μM of thyroid hormones present in the reaction mixture, with a decrease of inhibition along with decreasing thyroid hormone concentrations. (B) Total thyroid hormones T4 and T3 in plasma of male *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice fed a 60% HFD. Blood was collected terminally by heart puncture. No difference in total thyroid hormones could be observed between *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice.

Determination of body fat distribution and liver fat content in *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice by computed tomography

Of the mice on 60% high fat diet, we determined the body fat mass post mortem by computed tomography. By applying this method, we could discriminate subcutaneous from visceral fat to see potential differences in fat distribution. As shown in Fig. 8A, the animals had an average total fat content of 37% for *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ male mice, but we could not observe any difference between the two groups, neither in total adipose tissue nor in the distribution of visceral and subcutaneous fat. This result matched our expectations, since we

could not observe any difference in body weight in the animals tested. Additionally, we determined the liver fat content of *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice. As estimates of hepatic steatosis, liver/spleen ratio and liver-spleen difference are well recognised (37-38). Regarding diagnosis of steatosis, proposed cutoff values for human are $CT_{L/S}$ of 0.8 or CT_{L-S} of -9 with lower values indicating hepatic steatosis of 30% or greater (37). Our measurements resulted in the observation that all animals developed hepatic steatosis, however, no difference in degree could be observed between *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice.

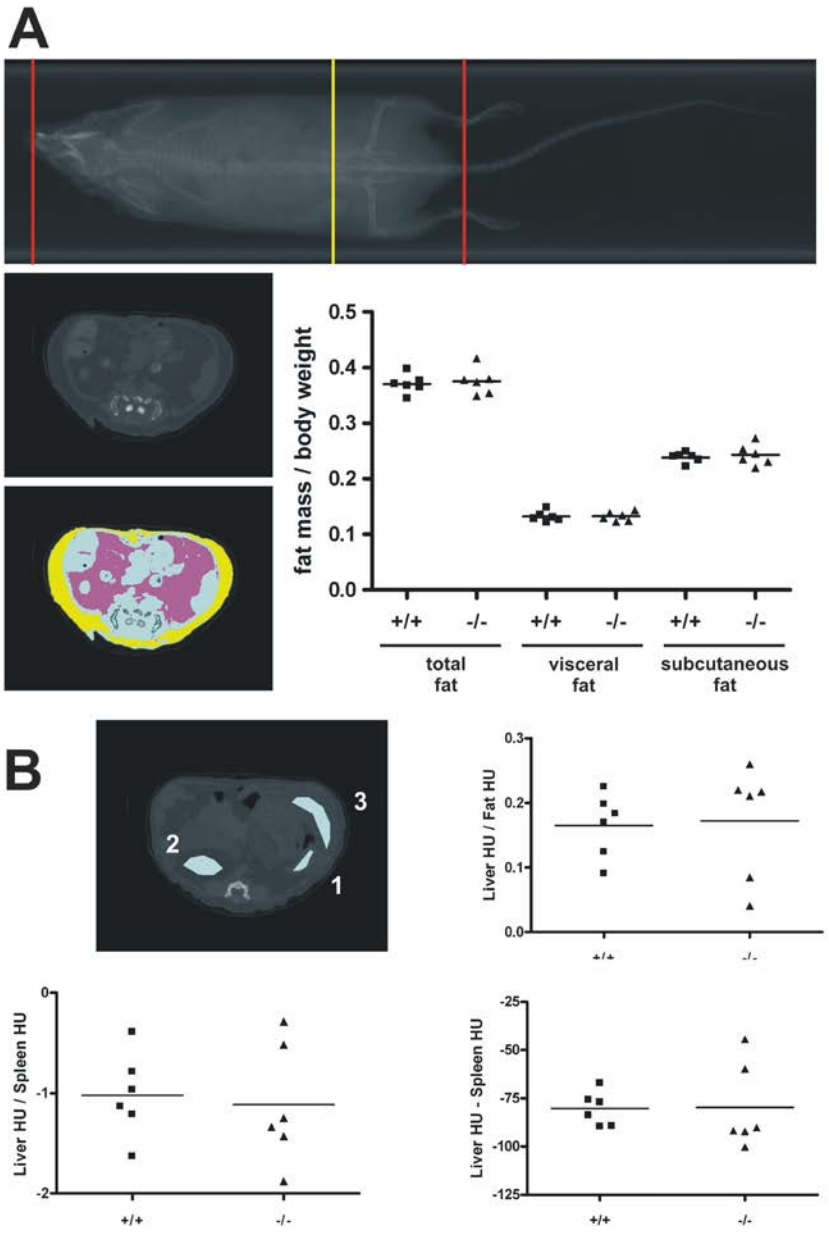


Fig. 8. Estimation of fat distribution and liver steatosis by computed tomography. (A) Mice were fed a high fat diet of 60% fat by calories for 16 weeks. Sacrified mice were scanned for analysis of visceral fat (purple) and subcutaneous fat (yellow) separated by the abdominal wall (lean mass, blue). No difference in total fat nor in fat distribution was detected. (B) Liver fat was estimated by computer tomography. Huntingfield units of liver (3) were compared to lean mass (spleen, 1) and visceral fat (2). Data plots show advanced steatosis for both *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice, but no significant difference in liver fat content was observed. See text for details.

DISCUSSION

When fed a high-fat diet (HFD), C57BL/6J mice are known to develop obesity and a series of symptoms reminiscent of the metabolic syndrome, including insulin resistance (27). A recent publication by the group of Jared Rutter suggested that *Paskin*$^{-/-}$ male mice are partially protected from the negative effects of a high fat diet, suggesting a hypermetabolic phenotype (39). We observed a similar trend that *Paskin*$^{-/-}$ mice are partially protected from the development of obesity under 45% high fat diet not only for male mice but also for females. However, feeding a high fat diet of 60% of fat by calories did not confirm the protective effect. As obesity results from the imbalance between energy intake and energy expenditure, metabolic cages were used to examine whether *Paskin*$^{-/-}$ mice were hypermetabolic. Rutter *et al.* found *Paskin*$^{-/-}$ mice to consume more oxygen, to emit more carbon dioxide and to generate more heat (18). We encountered another discrepancy to these findings when we applied indirect calorimetry to *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice. This discrepancy might be explained by the experimental setup, since the published phenotype was obtained from n=3 animals with an adaptation time to metabolic cages of 4 hours only (18), whereas our n=6 per genotype with 5 days of adaptation did not reveal any difference in indirect calorimetry nor body temperature. Interestingly, the stated partial protection from HFD induced obesity is combined with better blood glucose clearance for male *Paskin*$^{-/-}$ mice compared to wildtype mice. On one hand, the protection against obesity and the lowered bodyweight might consequently lead to better glucose clearance, on the other hand this phenotype is very common in a huge number of animal models. Mechanistical insights derived from these results could come from the observations by Rutter and colleagues that the ATP production rate was higher in *Paskin*$^{-/-}$ than in *Paskin*$^{+/+}$ soleus muscle fibres. However, neither an increase in mitochondrial number nor mass was observed that could account for this difference (18). A second finding of interest was the observation of increased fatty acid oxidation in L6 myoblasts with a PASKIN knockdown (18). Third, they found reduced triglyceride levels in the liver of *Paskin*$^{-/-}$ mice on HFD compared to *Paskin*$^{+/+}$ animals along with reduced levels of components involved in fatty acid synthesis and homeostasis. Although we could not confirm the protection from development of hepatic steatosis, we found increased levels of triglycerides in plasma of *Paskin*$^{+/+}$ animals but not in *Paskin*$^{-/-}$ samples.

On the cellular level, we suspected AMP kinase to be the responsible regulator of energy balance, influenced by PASKIN. AMPK can inhibit adipocyte lipolysis and stimulate fatty acid oxidation in many tissues, both acutely via phosphorylation of ACC2 (40) and chronically by upregulating PGC-1α (and thereby mitochondrial biogenesis) (41-42). These effects are protective against the development of insulin resistance and type 2 diabetes and could explain the increased levels of ATP in permeabilised soleus muscle, all of which are

published effects observed in *Paskin*$^{-/-}$ mice (18). On the other hand, hyperlipidemia leads to abnormal accumulation of triglycerides and fatty acids in muscle and liver that may disturb intracellular signaling pathways, leading to a decrease in insulin-stimulated glucose uptake (43). Furthermore, high levels of the saturated fatty acid palmitate affected the integrity of the endoplasmic reticulum *in vitro,* leading to cell death (44). When fatty acid oxidation was stimulated by pharmacological AMPK activation, this effect could be reversed. Whereas we found a tendency to elevated levels of phosphorylated (activated) AMPK (T172) in our mouse embryonic fibroblasts, this could not be confirmed in gastrocnemius extracts of *Paskin*$^{-/-}$ versus *Paskin*$^{+/+}$ mice. Also, we did not observe any difference in intracellular ATP levels in our wildtype versus knockout mouse embryonic fibroblasts. The fact that Rutter *et al.* did not observe a difference in phospho-AMPK in liver extracts (18) is supported by our gastrocnemius data, suggesting that PASKIN acts independently of AMPK. This still leaves the open question, how PASKIN could interfere with homeostatic patways. One potential explanation could be impaired insulin stimulated glucose uptake in peripheral tissues. The major glucose transporter in muscle and adipose tissues is GLUT4, a transporter stored intracellularly in vesicles that translocate to the cell membrane upon insulin stimulation. This step is considered as rate limiting and is severely disrupted in type 2 diabetes. A mouse model with muscle specific disruption of GLUT4 showed insulin resistance (45). On the other hand, overexpression of GLUT4 in the diabetic *db/db* mouse model partially restores the effects of the metabolic syndrome (46). Insulin dependent translocation to the outer cell membrane involves insulin receptor signaling via PI 3-kinase activating Akt and PKCζ. Self-evidently, the vesicular transport and membrane fusion machineries or retrograde vesicular recycling machineries are required (47). Several signalling pathways regulate the multiple cellular functions required for the functionality of insulin stimulated glucose uptake. How PASKIN might be involved in a negative regulatory manner in one or more of these functions that result in a potential metabolic phenotype for the *Paskin*$^{-/-}$ mice, remains to be invesitgated.

However, two findings may involve PASKIN in different signaling mechanisms. First, a knockdown of PASKIN in L6 muscle cells showed increased glucose and palmitate oxidation (18). Secondly, *Paskin*$^{-/-}$ livers show decreased triglyceride accumulation and a moderate downregulation for many genes involved in fatty acid uptake and synthesis with strong downregulation of fatty acid elongase, CD36 or PPARγ (18). Integrating these results into the phenotype of *Paskin*$^{-/-}$ mice also suggests a variety of potential regulatory functions of PASKIN. It is well known that insulin resistane may develop upon hyperlipidemia due to accumulation of fatty acid metabolites such as diacylglycerols and ceramides within insulin responsive tissues (48-49). Interestingly, for plasma of female *Paskin*$^{-/-}$ mice we saw increased levels of free fatty acids, which could be explained by the downregulation of fatty

acid transporter CD36 in *Paskin*$^{-/-}$ livers. The decreased uptake of fatty acids together with increased fatty acid oxidation in *Paskin*$^{-/-}$ liver or muscle could therefore protect from insulin resistance and hepatic steatosis. Interestingly, these effects are normally regulated by AMPK. Activated AMPK leads to decreased lipogenic gene expression and fatty acid synthesis and to increased fatty acid oxidation largely due to increased mitochondrial biogenesis (50). For high fat diet fed subjects, intracellular lipids increase β-oxidation (49). However, for untrained situations the oxidative capacity of muscles is not increased and will lead to a lipid overload that accumulates intermediate products derived from β-oxidation (48). Thus, induced mitochondrial stress is thought to activate stress kinases leading to insulin resistance apart from less 'active' mitochondria. As we reported herein, that *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice do not differ in volontary exercise using running wheels. It would be interesting to see whether *Paskin*$^{-/-}$ mice fed a high fat diet would show a better performance in forced running on a treadmill, both acutely as well as in physical exercise training to substantiate a role for PASKIN regulating lipid flux and mitochondrial stress response. Additionnal reasons for such experiments are contributed by the finding that PASKIN has also been shown to phosphorylate glycogen synthase (14), what could lead to a breakdown of cellular glycogen storage and could display a phenotype in muscle and liver.

The different outcomes of the animal experiments in two different laboratories might point to subtle experimental parameters such as diet or housing that might differ. However, since we lack mechanistic explanations for a PASKIN function in energy homeostasis on a cellular level, it is difficult to track down the origins of the discrepancies. Further experiments on lipid metabolism in adipose tissue will certainly shed light on open questions regarding the *Paskin*$^{-/-}$ phenotype on HFD. Finally, the identification of a potential activating (metabolic) ligand for PASKIN is long sought-for and would most certainly help to integrate PASKIN into the complex system of metabolic homeostasis.

REFERENCES

1 Taylor BL, Zhulin IB (1999) PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol Mol Biol Rev* **63**: 479-506.

2 Schlafli P, Borter E, Spielmann P, Wenger RH (2009) The PAS-domain kinase PASKIN: a new sensor in energy homeostasis. *Cell Mol Life Sci* **66**: 876-883.

3 Mimura J, Fujii-Kuriyama Y (2003) Functional role of AhR in the expression of toxic effects by TCDD. *Biochim Biophys Acta* **1619**: 263-268.

4 Lendahl U, Lee KL, Yang H, Poellinger L (2009) Generating specificity and diversity in the transcriptional response to hypoxia. *Nat Rev Genet* **10**: 821-832.

5 Wenger RH (2002) Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. *Faseb J* **16**: 1151-1162.

6 Reppert SM, Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* **418**: 935-941.

7 Hofer T, Spielmann P, Stengel P, Stier B, Katschinski DM, Desbaillets I, Gassmann M, Wenger RH (2001) Mammalian PASKIN, a PAS-serine/threonine kinase related to bacterial oxygen sensors. *Biochem Biophys Res Commun* **288**: 757-764.

8 Rutter J, Michnoff CH, Harper SM, Gardner KH, McKnight SL (2001) PAS kinase: an evolutionarily conserved PAS domain-regulated serine/threonine kinase. *Proc Natl Acad Sci U S A* **98**: 8991-8996.

9 Amezcua CA, Harper SM, Rutter J, Gardner KH (2002) Structure and interactions of PAS kinase N-terminal PAS domain: model for intramolecular kinase regulation. *Structure* **10**: 1349-1361.

10 Katschinski DM, Marti HH, Wagner KF, Shibata J, Eckhardt K, Martin F, Depping R, Paasch U, Gassmann M, Ledermann B, Desbaillets I, Wenger RH (2003) Targeted disruption of the mouse PAS domain serine/threonine kinase PASKIN. *Mol Cell Biol* **23**: 6780-6789.

11 Borter E, Niessen M, Zuellig R, Spinas GA, Spielmann P, Camenisch G, Wenger RH (2007) Glucose-stimulated insulin production in mice deficient for the PAS kinase PASKIN. *Diabetes* **56**: 113-117.

12 Eckhardt K, Troger J, Reissmann J, Katschinski DM, Wagner KF, Stengel P, Paasch U, Hunziker P, Borter E, Barth S, Schlafli P, Spielmann P, Stiehl DP, Camenisch G, Wenger RH (2007) Male germ cell expression of the PAS domain kinase PASKIN and its novel target eukaryotic translation elongation factor eEF1A1. *Cell Physiol Biochem* **20**: 227-240.

13 Rutter J, Probst BL, McKnight SL (2002) Coordinate regulation of sugar flux and translation by PAS kinase. *Cell* **111**: 17-28.

14 Wilson WA, Skurat AV, Probst B, de Paoli-Roach A, Roach PJ, Rutter J (2005) Control of mammalian glycogen synthase by PAS kinase. *Proc Natl Acad Sci U S A* **102**: 16596-16601.

15 da Silva Xavier G, Rutter J, Rutter GA (2004) Involvement of Per-Arnt-Sim (PAS) kinase in the stimulation of preproinsulin and pancreatic duodenum homeobox 1 gene expression by glucose. *Proc Natl Acad Sci U S A* **101**: 8319-8324.

16 Fontes G, Semache M, Hagman DK, Tremblay C, Shah R, Rhodes CJ, Rutter J, Poitout V (2009) Involvement of Per-Arnt-Sim Kinase and extracellular-regulated kinases-1/2 in palmitate inhibition of insulin gene expression in pancreatic beta-cells. *Diabetes* **58**: 2048-2058.

17 An R, da Silva Xavier G, Hao HX, Semplici F, Rutter J, Rutter GA (2006) Regulation by Per-Arnt-Sim (PAS) kinase of pancreatic duodenal homeobox-1 nuclear import in pancreatic beta-cells. *Biochem Soc Trans* **34**: 791-793.

18 Hao HX, Cardon CM, Swiatek W, Cooksey RC, Smith TL, Wilde J, Boudina S, Abel ED, McClain DA, Rutter J (2007) PAS kinase is required for normal cellular energy balance. *Proc Natl Acad Sci U S A* **104**: 15466-15471.

19 Kahn BB, Alquier T, Carling D, Hardie DG (2005) AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* **1**: 15-25.

20 Hardie DG, Carling D (1997) The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* **246**: 259-273.

21 Stroka DM, Burkhardt T, Desbaillets I, Wenger RH, Neil DA, Bauer C, Gassmann M, Candinas D (2001) HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *Faseb J* **15**: 2445-2453.

22 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.

23 Cavadini G, Petrzilka S, Kohler P, Jud C, Tobler I, Birchler T, Fontana A (2007) TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription. *Proc Natl Acad Sci U S A* **104**: 12843-12848.

24 Weir JB (1949) New methods for calculating metabolic rate with special reference to protein metabolism. *J Physiol* **109**: 1-9.

25 Hillebrand JJ, Langhans W, Geary N (2009) Validation of computed tomographic estimates of intra-abdominal and subcutaneous adipose tissue in rats and mice. *Obesity (Silver Spring)* **18**: 848-853.

26 Piekarski J, Goldberg HI, Royal SA, Axel L, Moss AA (1980) Difference between liver and spleen CT numbers in the normal adult: its usefulness in predicting the presence of diffuse liver disease. *Radiology* **137**: 727-729.

27 Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feinglos MN (1988) Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* **37**: 1163-1167.

28 Spiegelman BM, Flier JS (2001) Obesity and the regulation of energy balance. *Cell* **104**: 531-543.

29 Stanley PE, Williams SG (1969) Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anal Biochem* **29**: 381-392.

30 Healy DA, Watson RW, Newsholme P (2002) Glucose, but not glutamine, protects against spontaneous and anti-Fas antibody-induced apoptosis in human neutrophils. *Clin Sci (Lond)* **103**: 179-189.

31 Hsu CL, Yen GC (2007) Effect of gallic acid on high fat diet-induced dyslipidaemia, hepatosteatosis and oxidative stress in rats. *Br J Nutr* **98**: 727-735.

32 Yuan G, Al-Shali KZ, Hegele RA (2007) Hypertriglyceridemia: its etiology, effects and treatment. *Cmaj* **176**: 1113-1120.

33 Marques CM, Motta VF, Torres TS, Aguila MB, Mandarim-de-Lacerda CA (2010) Beneficial effects of exercise training (treadmill) on insulin resistance and nonalcoholic fatty liver disease in high-fat fed C57BL/6 mice. *Braz J Med Biol Res* **43**: 467-475.

34 Larsen F, Rossner S (1983) Serum creatine kinase in obese subjects before and during weight reduction. *Clin Chim Acta* **133**: 285-288.

35 Reyns GE, Venken K, Morreale de Escobar G, Kuhn ER, Darras VM (2003) Dynamics and regulation of intracellular thyroid hormone concentrations in embryonic chicken liver, kidney, brain, and blood. *Gen Comp Endocrinol* **134**: 80-87.

36 Silva JE (2006) Thermogenic mechanisms and their hormonal regulation. *Physiol Rev* **86**: 435-464.

37 Park SH, Kim PN, Kim KW, Lee SW, Yoon SE, Park SW, Ha HK, Lee MG, Hwang S, Lee SG, Yu ES, Cho EY (2006) Macrovesicular hepatic steatosis in living liver donors: use of CT for quantitative and qualitative assessment. *Radiology* **239**: 105-112.

38 Mehta SR, Thomas EL, Bell JD, Johnston DG, Taylor-Robinson SD (2008) Non-invasive means of measuring hepatic fat content. *World J Gastroenterol* **14**: 3476-3483.

39 Hao HX, Rutter J (2008) The role of PAS kinase in regulating energy metabolism. *IUBMB Life* **60**: 204-209.

40 Merrill GF, Kurth EJ, Hardie DG, Winder WW (1997) AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol* **273**: E1107-1112.

41 Terada S, Goto M, Kato M, Kawanaka K, Shimokawa T, Tabata I (2002) Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. *Biochem Biophys Res Commun* **296**: 350-354.

42 Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, Shulman GI (2002) AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proc Natl Acad Sci U S A* **99**: 15983-15987.

43 Lowell BB, Shulman GI (2005) Mitochondrial dysfunction and type 2 diabetes. *Science* **307**: 384-387.

44 Borradaile NM, Han X, Harp JD, Gale SE, Ory DS, Schaffer JE (2006) Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. *J Lipid Res* **47**: 2726-2737.

45 Stenbit AE, Tsao TS, Li J, Burcelin R, Geenen DL, Factor SM, Houseknecht K, Katz EB, Charron MJ (1997) GLUT4 heterozygous knockout mice develop muscle insulin resistance and diabetes. *Nat Med* **3**: 1096-1101.

46 Brozinick JT, Jr., McCoid SC, Reynolds TH, Nardone NA, Hargrove DM, Stevenson RW, Cushman SW, Gibbs EM (2001) GLUT4 overexpression in db/db mice dose-dependently ameliorates diabetes but is not a lifelong cure. *Diabetes* **50**: 593-600.

47 Bryant NJ, Govers R, James DE (2002) Regulated transport of the glucose transporter GLUT4. *Nat Rev Mol Cell Biol* **3**: 267-277.

48 Muoio DM, Koves TR (2007) Skeletal muscle adaptation to fatty acid depends on coordinated actions of the PPARs and PGC1 alpha: implications for metabolic disease. *Appl Physiol Nutr Metab* **32**: 874-883.

49 Samuel VT, Petersen KF, Shulman GI (2010) Lipid-induced insulin resistance: unravelling the mechanism. *Lancet* **375**: 2267-2277.

50 Hegarty BD, Turner N, Cooney GJ, Kraegen EW (2009) Insulin resistance and fuel homeostasis: the role of AMP-activated protein kinase. *Acta Physiol (Oxf)* **196**: 129-145.

9 MANUSCRIPT V (UNPUBLISHED)

PASKIN is predominantly expressed in leukocytes and is localised along the actin cytoskeleton.

Philipp Schläfli, Sascha Egger, Flurin Item, Patrick Spielmann and Roland H. Wenger

Institute of Physiology and Zürich Center for Integrative Human Physiology ZIHP, University of Zürich UZH, CH-8057 Zürich, Switzerland

ABSTRACT

PASKIN is a PAS domain containing serine/threonine kinase which is ubiquitously expressed at low levels throughout the body with particularly high expression in testis. The yeast orthologs of PASKIN have been shown to be involved in protein translation and glucose homeostasis. Recent findings have confirmed PASKIN as a regulatory kinase in mammals. Accordingly, PASKIN has been reported to be involved in the regulation of insulin expression in the β-cells of pancreatic islets of Langerhans as well as in the secretion of glucagon in α-cells. Furthermore, *Paskin*$^{-/-}$ mice have been shown to be partially protected from obesity and detrimental effects of the metabolic syndrome upon feeding a high fat diet. However, the pathways conferring these phenotypes remain unclear. A current model for PASKIN activation speculates about a small endogenous ligand binding to the PAS domain and de-repressing the kinase domain. Recently, we identified monophosphorylated phosphatidylinositides as potential ligands of PASKIN. This encouraged us to investigate PASKIN expression in different cell lines and to determine its subcellular localisation.

INTRODUCTION

Per-ARNT-Sim (PAS) domains are widespread in sensory proteins of bacteria and archaea (1). In nitrogen fixating bacteria of *Rhizobium* and *Bradyrhizobium* species the oxygen sensing protein FixL contains a heme moiety bound to the PAS domain where oxygen binds to ferrous iron, induces conformational changes and inhibits the histidine kinase domain (2). In contrast, under oxygen-free conditions, the kinase is de-repressed and induces transcription of genes involved in nitrogen fixation. We and others identified a mammalian PAS protein with a highly similar PAS domain to FixL (*3-4*). The two N-terminal PAS domains of this protein were linked to a serine/threonine kinase domain, and therefore this protein was named PASKIN or PAS kinase. Similar to FixL, a current model for PASKIN function suggests, that the PAS domain of PASKIN requires binding of a small endogenous ligand to activate the kinase by de-repression (5). Functionally the yeast orthologs Psk1 and Psk2 have been shown to regulate glycogen and trehalose synthesis and phosphorylate three translation factors, implicating a role for Psk proteins in linking protein translation and energy homeostasis (6).
Using a human tissue array, we found that PASKIN mRNA was ubiquitously expressed with particulary high levels in testis and brain (3). These results were confirmed by quantitative PCR of mouse tissue samples, where the expression levels in testis were 100-fold higher than in any other tissue, but showed moderately high expression in brain, bone marrow and thymus, as well (7). Functionally, most findings were derived from pancreatic α- and β-cells, where PASKIN has been reported to be involved in the upregulation of insulin (*8-9*) and inhibited secretion of glucagon (*10*). Furthermore, we previously generated *Paskin*$^{-/-}$ mice by replacing the kinase domain of PASKIN with a *lacZ-neo* fusion construct, resulting in a mouse model with no apparent phenotype under standard conditions (11). However, when fed a high fat diet, these mice were reported to be partially protected from obesity and detrimental effects of the metabolic syndrome, including insulin resistance (12). Yet, we could only confirm some of the results of the knock-out animals (13). However, all results involving PASKIN in metabolism still lack mechanistical explanations and no activating small molecular weight ligand has been identified. Because we found that PASKIN binds to monophosphorylated phosphatidylinositides (submitted), we were interested, in what cell types and cell culture models PASKIN is expressed. Phosphatidylinositides are mainly regulated by lipid kinases and phosphatases and provide hallmarks for intracellular compartments (14). Whereas PtdIns(4)P is predominantly localised at the Golgi apparatus, PtdIns(3)P is enriched in vesicles of the endosomal compartment. We were therefore interested in the subcellular localisation of PASKIN in different cell models to find links to phosphatidylinositide binding. This might improve our understanding of potential functions of

PASKIN and reveal further information about the potential mechanistics in metabolic homeostasis.

MATERIALS AND METHODS

Cell culture

HeLa, Hek293, HepG2, MEFs and C2C12 myoblasts were cultured in Dulbecco's modified Eagle's media (Sigma, D6429) containing 10% FCS; 33.1.1, K-46 and HL-60 were cultured in Iscove's modified Dulbecco's media (Sigma, I3390) with 10% FCS; Jurkat, THP-1 and RAW264.7 were cultured in RPMI 1640 (Sigma, R8758) with 10% FCS. C2C12 myoblasts were differentiated to myotubes at approximately 80% confluency by changing media to DMEM supplied with 2% horse serum for 4 days, changing the media every 24 hours. AB81 cells were grown as described elsewhere (15) in RPMI 1640 (Gibco, 52400) containing 10% FCS and insulin-transferrin-selenite supplement (Roche Applied Sciences) at 33°C and differentiated at 80% confluency by changing incubation temperature to 37°C for 60 hours.

Immunoblotting

Cells were harvested and whole cell lysates were generated by heating the cells in PBS/1% SDS/50 mM β-mercaptoethanol for 5 minutes at 95°C as described previously (16). Concentrations of supernatants were determined by Bradford assay (17). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences). For immunoblot analysis, anti-mPASK (Cell signaling) and anti-β-actin (Sigma) were used. As secondary antibodies, polyclonal goat anti-mouse or anti-rabbit antibodies coupled to horseradish peroxidase (Pierce) were used and chemiluminescence was recorded with a CCD camera (LAS-4000, Fujifilm).

mRNA quantification

Total RNA from cultured cells was isolated as described previously (18). Human PASKIN, human L28, mouse PASKIN and mouse S12 mRNA were determined by RT-qPCR. RNA was reverse transcribed with StrataScript III (Stratagene) and qPCR was performed using a SYBRGreen qPCR reagent kit (Sigma) on a MX3000P PCR light cycler (Stratagene) according to the manufacturer's instructions. Primers (Microsynth, Switzerland) used were: hPASKINfwd 5'-ggaactgctccagtttcctg-3' and hPASKINrev 5'-ggatctccgcttaatgacca-3'; mPASKINfwd 5'-agggtccaagaattgacgtg-3' and mPASKINrev 5'-tgactgctcaccatcctctg-3'; ribosomal protein L28, hL28fwd 5'-ggaactgctccagtttcctg-3' and hL28rev 5'-ggatctccgcttaatgacca-3'; mouse ribosomal protein S12, mS12fwd 5'-gaagctgccaaagccttaga-3';

mS12rev 5'aactgcaaccaaccaccttc-3' Dilution series of the corresponding gel-isolated PCR products were used to obtain standard curves.

Skeletal muscle biopsies

Informed written consent was obtained from donors. The experimental protocol was approved by the ethics committee of the canton of Zurich in accordance with the ethical standards laid down in the Declaration of Helsinki for human experimentation. Percutaneous biopsies from the middle region of the nondominant *vastus lateralis* muscle were obtained using a 6-mm Bergström needle (Dixons Surgical Instruments, Essex, UK) with suction applied and immediately mounted in embedding medium (Tissue-Tek®, Sakura) and snap frozen in isopentane cooled with liquid nitrogen for subsequent cryosectionning at 12 μm and immunofluorescence analysis.

Laser scanning fluorescence microscopy

Immunofluorescence stainings of cells were performed as described previously (19). Cells were cultivated on microscope coverslips, washed twice with ice-cold PBS, fixed on ice for 30 min with 4% paraformaldehyde, and permeabilized with 0.1% saponin (Merck) in PBS. PASKIN was detected using anti-PASKIN antibody (ABR) and visualized with goat anti-mouse AlexaFluor-488 (Molecular Probes, Invitrogen). F-actin was stained using AlexaFluor-635 phalloidin (Molecular Probes, Invitrogen) and 4′,6-diamidino-2-phenylindole (Sigma) was used to stain nulei. The slides were embedded in Mowiol (Calbiochem) and analyzed by confocal laser scanning microscopy (SP2, Leica Microsystems). *In silico* analysis of colocalisation was performed using Imaris 7.0 software (Bitplane).

RESULTS

Basal expression levels of PASKIN are elevated in lymphocytes

A high expression of PASKIN in testis and brain has been repeatedly reported (3,7,11). However, previously reported functions for PASKIN in metabolism have been derived from observations in cell culture models from pancreatic cells, i.e. the mouse β-cell line Min6 (*8-9*) and the human α-cell line alpha-TC1-9 (20) as well as L6 rat skeletal muscle cells (*12*). To find other potential cell culture models, we performed a database search using Gene Atlas (http://biogps.gnf.org). In the probe set of a human Affymetrix U133 array that includes 39.000 hybridisation probes, PASKIN expression in different organs is shown (Fig. 1). Interestingly, the highest expression of PASKIN was not observed in testis, even though it

was among the strongest expressing (3 times over median), but in lymphatic cells, especially CD8+ cytotoxic T-cells and CD4+ T-helper cells laying >40 times over the median.

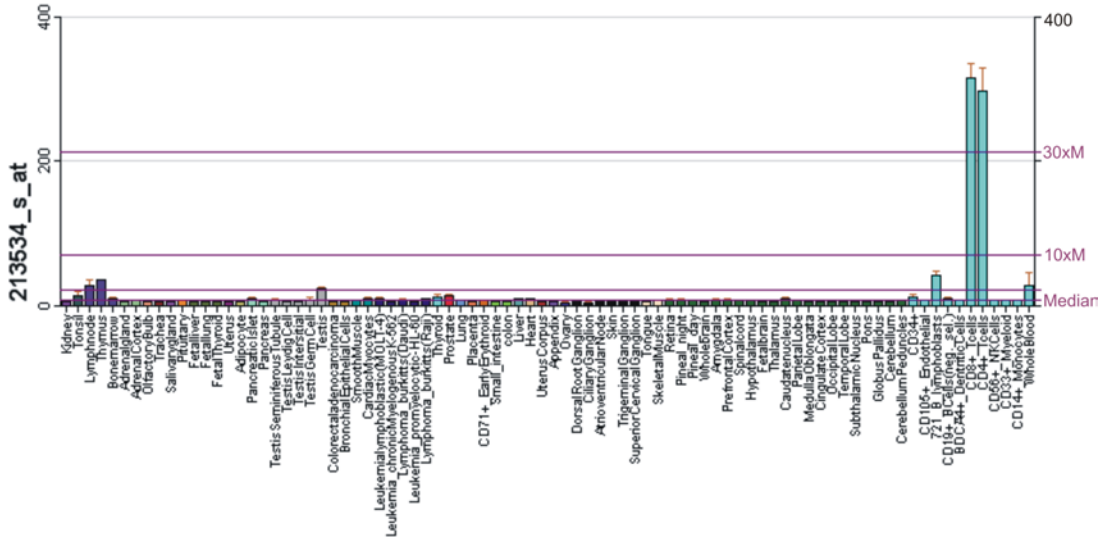


Fig. 1. PASKIN expression in different human tissues. The database entry for the oligo 213534_s_at from the human affymetrix chip U133A for PASKIN for different organ samples shows increased expression in lymphatic cells and organs, whereas the previously reported primary site of PASKIN expression, the testis, only appear 3-fold over median.

Intrigued by this finding, we were interested to test various established human and mouse cell lines for PASKIN mRNA and protein expression, focusing on lymphoid and myeloid cell types. For this purpose, we chose six human cell lines (Fig. 2A/B) and seven mouse cell lines (Fig. 2C/D).

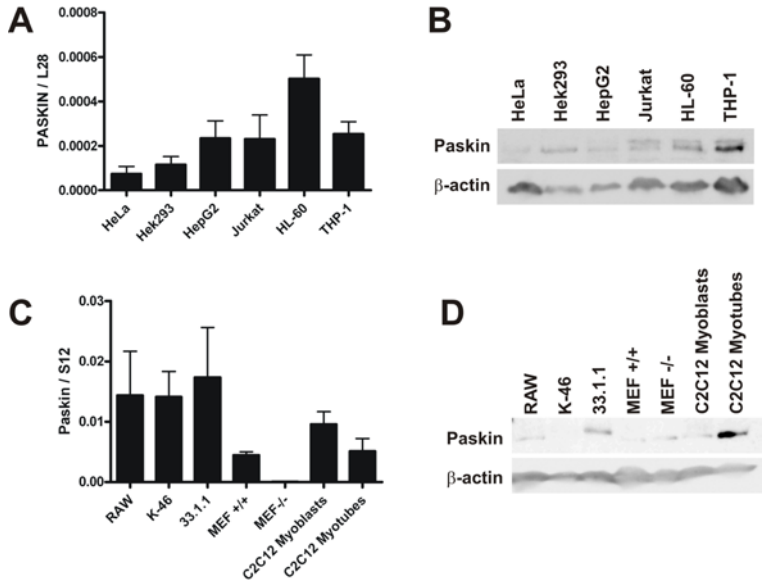


Fig. 2. PASKIN mRNA and protein levels in various cell lines. Cell lines tested were analysed by quantitative real time PCR (A, B) or Western blot using an antibody directed against mouse Paskin with crossreactivity to human PASKIN (C). Shown are mean&SD of n=3 independent experiments for mRNA levels and a representative Western blot.

Expression of PASKIN mRNA in human cell lines indeed revealed strongest expression in HL-60, a neutrophilic promyelocyte cell line and more than two fold higher expression for the T-cell leukemic Jurkat cells and THP-1 monocytes than for HeLa and Hek293. Confirming this result, we observed the highest expression of PASKIN in mouse cell lines for the 33.1.1 pre-B cell line, K-46 B-cells and RAW264.7 macrophages. Regarding protein expression levels, the differences in mRNA levels appeared to be blunted but keeping elevated PASKIN levels in THP-1 monocytes, 33.1.1 pre-B cells and surprisingly for differentiated C2C12 myofibers. We tested whether PASKIN mRNA levels were affected when the lymphoid and myeloid cell lines were treated with lipopolysaccharides (LPS) as proinflammatory stimulus. However, mRNA levels appeared not to be induced (data not shown).

PASKIN is localised along the F-actin cytoskeleton

As we found that PASKIN binds to monophosphorylated phosphatidylinositides (manuscript in preparation), we were interested in the subcellular localisation of PASKIN. For this purpose, we performed immunofluorescence experiments in HeLa cells, a cell line showing moderate PASKIN expression with the advantage of being adherent cells with a defined cellular structure compared to suspension cell lines.

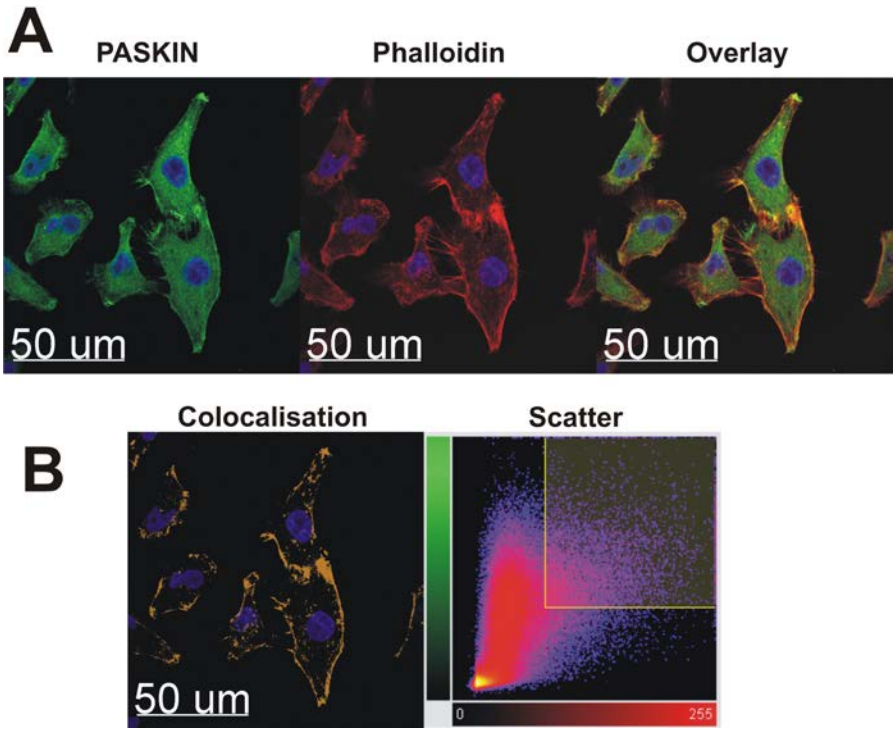


Fig. 3. PASKIN co-localises with filamentous actin at the edges of HeLa cells. PASKIN subcellular localisation was determined by confocal laser scanning microscopy on HeLa cells co-stained with phalloidin (stain for filamentous actin) (A). A strong signal for PASKIN and co-localisation occurred mainly at the edges of the cells, potentially representing focal adhesion sites. (B) The scatter plot shows the intensity distribution for the PASKIN (green) and the phalloidin (red) channel. From this plot, the region with the strongest intensities in both channels was selected (shaded square) to generate the artificial co-localisation image confirming a strong staining at the edges of the cells.

Interstingly, when applying confocal microscopy focusing to the lowest layers of the cell, we observed a strong staining for focal adhesion sites and filopodial structures at the edges of the cells correlating with the signal for the phalloidin staining for filamentous actin (Fig.3). However, as expected for a kinase and published previously, PASKIN could also be detected in the cytoplasm (21). To confirm the signal in filopodial structures we aimed for staining of another cell line, namely the AB-81 podocyte cell line that proliferates at 33°C growth temperature and differentiates to podocytes at 37°C forming cytoplasmic extensions of lamellipodial character (15). However, whereas PASKIN was not explicitly expressed at cell edges, we observed PASKIN accumulation along the F-actin cytoskeleton in this cell line (Fig.4).

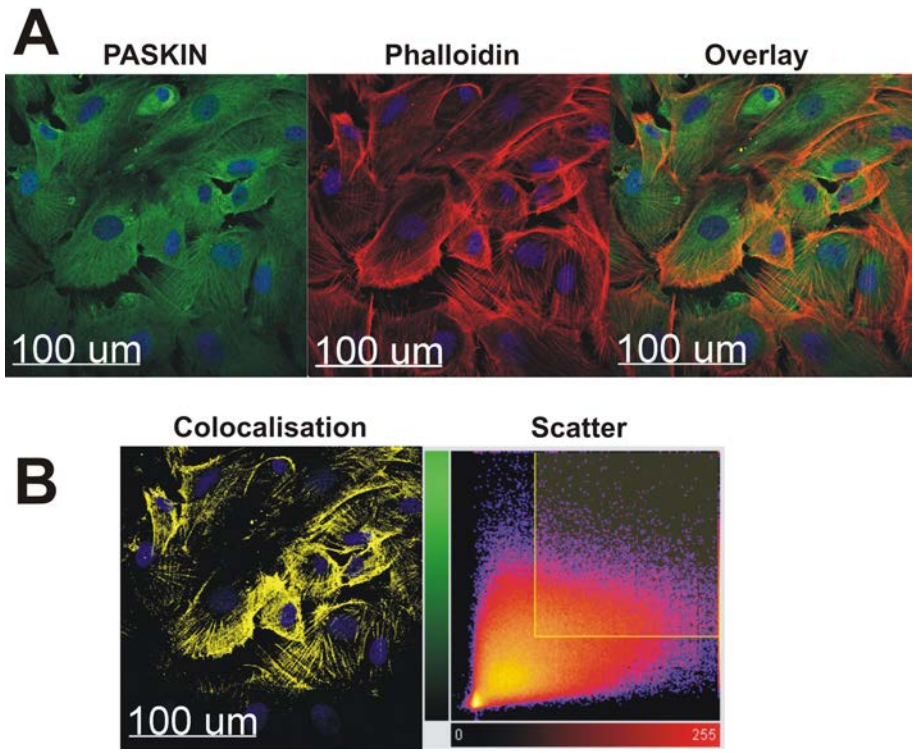


Fig. 4. PASKIN localises along the actin cytoskeleton in differentiated AB-81 podocyte cells. PASKIN subcellular localisation was determined by confocal microscopy on differentiated AB-81 cells, immuno-stained for PASKIN and phalloidin (A). Colocalisation occurred along the filamentous actin cytoskeleton. Similar to Fig. 3, a co-localisation channel was generated (B).

To find a physiological link between F-actin co-localisation with PASKIN and a potential metabolic function, we stained human *vastus lateralis* skeletal muscle cross-sections. Interestingly, PASKIN seems to be highly expressed in skeletal muscle fibres compared to the surrounding connective tissue (Fig. 5), supporting the elevated expression in C2C12 myotubes (Fig. 2). The distribution of PASKIN within the *vastus lateralis* fibres resembles the distribution of myosin heavy chain that is used to determine the fiber types (22).

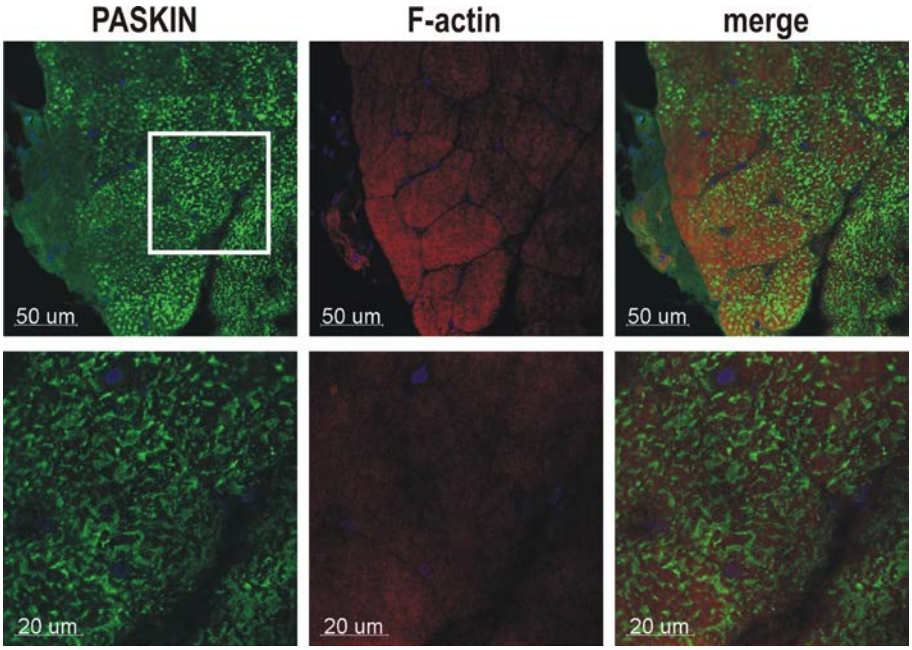


Fig. 5. PASKIN expression in human skeletal muscle cross-sections. Cross-sections from human *vastus lateralis* muscle were stained for PASKIN and phalloidin. The staining resembles published against myosin heavy chain isoform staining which are used to determine the fibre type of skeletal muscles (22).

DISCUSSION

We herein aimed to answer two major questions. First, we were interested in identifying cell lines of human origin that show elevated basal levels of PASKIN expression, as previously published findings are all based on either mouse or rat cell lines or primary human material. Secondly, we aimed to investigate the subcellular localisation of PASKIN, since we recently discovered that PASKIN binds to phosphatidylinositol monophosphates (submitted).

Using the Gene Atlas, we were surprised to find high PASKIN expression in lymphatic cells, as PASKIN has not been involved into processes of the immune response before. Indeed, testing immune cell lines for mRNA and protein expression confirmed this finding. As we yet lack information, how PASKIN expression is regulated, activated and what might be the potential intracellular ligand for the PAS domain, it was intriguing to test how PASKIN expression levels were altered when lymphatic cells were stimulated. Therefore we used classical Toll like receptor 4 activating lipopolysaccharides (LPS). However, we did not find any differences in PASKIN expression levels after treatment (data not shown).

To address the question of subcellular localisation, we switched the model from immune cells to HeLa. Interestingly, we found PASKIN to be localised at the edges of the cell in structures resembling to focal adhesion sites. The co-staining with phalloidin revealed that PASKIN co-localises with filamentous actin at the lower layers scanned, and was also slightly enriched in filopodial structures. To confirm this finding, we were interested in the expression levels of

PASKIN in AB-81, a podocyte cell line of human origin that proliferates at 33°C and differentiates at 37°C forming lammellipodia-like structures. Interestingly, we found PASKIN localisation in differentiated (and also undifferentiated cells, data not shown) AB-81 cells along the filamentous actin cytoskeleton rather than at the cell periphery. Podocytes are known for strongly remodelling thecytoskeleton which defines their cell shape, mediates cell-cell contacts in the glomeruli and might even contract upon shear stress induced by hydraulic pressure. This enables podocytes to control the rate of filtration within glomeruli (23). Further experiments might disclose the involvement of PASKIN in F-actin reorganisation or vesicular trafficking along the filaments.

Another potential function would explain our recent finding that PASKIN binds to PtdIns(3)P, PtdIns(4)P and PtdIns(5)P. As especially PtdIns(4)P is formed in the Golgi apparatus and PtdIns(3)P is enriched on vesicular and endosomal structures of cells (14), the association of PASKIN to the cytoskeleton might represent the *in vivo* situation of phosphatidylinositide binding.

F-actin cytoskeleton association or processes involving membrane dynamics and sorting may also help to explain several metabolic functions of PASKIN. We previously reported that the elongation factor eEF1A1 is a target of PASKIN (21), and it is interesting to note that eEF1A1 was shown to bind filamentous actin directly and colocalises with phalloidin staining at the cellular membrane (24). Therefore, it is hypothesized that eEF1A1 and other actin-binding components of the translational machinery might concentrate along the cytoskeleton to optimise translation in a spatially restricted manner (25). Other reports on PASKIN function in higher organisms speculate about an inhibitory role for PASKIN in glucagon secretion in pancreatic α-cells (*10*), or an activating role in insulin secretion in β-cells (*8*), even though we published conflicting results (*7*). As secretion of insulin and glucagon granules require an intact pool of PtdIns(4,5)P_2 (26-28), it is tempting to speculate that PASKIN might influence the precursors PtdIns, PtdIns(4)P or PtdIns(5)P, and that this could explain the moderate effects that might lead to conflicting results between our lab and the results published by da Silva Xavier *et al.* (*8*).

Using our *Paskin*$^{-/-}$ mouse model, peripheral effects of glucose homeostasis have been reported as well. When fed a high fat diet, *Paskin*$^{-/-}$ mice seem to be protected from detrimental effects of the metabolic syndrome with less glucose intolerance (*12*). As we herein present a robust expression of PASKIN in *vastus lateralis* skeletal muscle cross-sections, a potential regulatory function in glucose uptake might originate from the secretion of GLUT4 containing vesicles. Indeed, GLUT4 translocation not only relies on the formation of PtdIns(3,4,5)P_3 by PI 3-kinase but also involves other phosphatidylinositides such as the PASKIN binding PtdIns(3)P, PtdIns(4)P and PtdIns(5)P that seem to be important for vesicle formation and vesicular transport (29). As *Paskin*$^{-/-}$ mice showed improved blood glucose

uptake in both glucose and insulin tolerance tests, PASKIN might play an inhibitory role for GLUT4 containing vesicle exocytosis by modulating processes of vesicle formation, transport or fusion via phosphatidylinositide signalling. Most interestingly, PASKIN staining of the *vastus lateralis* skeletal muscle revealed a picture resembling to myosin staining (22). Myo1C can be phosphorylated by CaMKII upon insulin stimulation and has been reported to enhance GLUT4 translocation (30-31), and therefore, it might be interesting to investigate, whether PASKIN, itself a member of the CaM kinase superfamily, might influence GLUT4 translocation as well. Staining of GLUT4 in insulin stimulated myocytes of *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice should clarify such a role for PASKIN in glucose uptake.

Conclusively, the results herein might present a link between phosphatidylinositide signalling and subcellular localisation. The association of PASKIN to the F-actin cytoskeleton provides new insight in potential cellular mechanistics that to date are unknown for PASKIN. Future research should also clarify, whether metabolic phenotypes for Paskin$^{-/-}$ mice might derive from phosphatidylinositide-signalling dependent effects.

REFERENCES

1 Taylor BL, Zhulin IB (1999) PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol Mol Biol Rev* **63**: 479-506.

2 Fischer HM (1994) Genetic regulation of nitrogen fixation in rhizobia. *Microbiol Rev* **58**: 352-386.

3 Hofer T, Spielmann P, Stengel P, Stier B, Katschinski DM, Desbaillets I, Gassmann M, Wenger RH (2001) Mammalian PASKIN, a PAS-serine/threonine kinase related to bacterial oxygen sensors. *Biochem Biophys Res Commun* **288**: 757-764.

4 Rutter J, Michnoff CH, Harper SM, Gardner KH, McKnight SL (2001) PAS kinase: an evolutionarily conserved PAS domain-regulated serine/threonine kinase. *Proc Natl Acad Sci U S A* **98**: 8991-8996.

5 Amezcua CA, Harper SM, Rutter J, Gardner KH (2002) Structure and interactions of PAS kinase N-terminal PAS domain: model for intramolecular kinase regulation. *Structure* **10**: 1349-1361.

6 Rutter J, Probst BL, McKnight SL (2002) Coordinate regulation of sugar flux and translation by PAS kinase. *Cell* **111**: 17-28.

7 Borter E, Niessen M, Zuellig R, Spinas GA, Spielmann P, Camenisch G, Wenger RH (2007) Glucose-stimulated insulin production in mice deficient for the PAS kinase PASKIN. *Diabetes* **56**: 113-117.

8 da Silva Xavier G, Rutter J, Rutter GA (2004) Involvement of Per-Arnt-Sim (PAS) kinase in the stimulation of preproinsulin and pancreatic duodenum homeobox 1 gene expression by glucose. *Proc Natl Acad Sci U S A* **101**: 8319-8324.

9 Fontes G, Semache M, Hagman DK, Tremblay C, Shah R, Rhodes CJ, Rutter J, Poitout V (2009) Involvement of Per-Arnt-Sim Kinase and extracellular-regulated kinases-1/2 in palmitate inhibition of insulin gene expression in pancreatic beta-cells. *Diabetes* **58**: 2048-2058.

10 da Silva Xavier G, Farhan H, Kim H, Caxaria S, Johnson P, Hughes S, Bugliani M, Marselli L, Marchetti P, Birzele F, Sun G, Scharfmann R, Rutter J, Siniakowicz K, Weir G, Parker H, Reimann F, Gribble FM, Rutter GA Per-arnt-sim (PAS) domain-containing protein kinase is downregulated in human islets in type 2 diabetes and regulates glucagon secretion. *Diabetologia*.

11 Katschinski DM, Marti HH, Wagner KF, Shibata J, Eckhardt K, Martin F, Depping R, Paasch U, Gassmann M, Ledermann B, Desbaillets I, Wenger RH (2003) Targeted disruption of the mouse PAS domain serine/threonine kinase PASKIN. *Mol Cell Biol* **23**: 6780-6789.

12 Hao HX, Cardon CM, Swiatek W, Cooksey RC, Smith TL, Wilde J, Boudina S, Abel ED, McClain DA, Rutter J (2007) PAS kinase is required for normal cellular energy balance. *Proc Natl Acad Sci U S A* **104**: 15466-15471.

13 Schlafli P, Borter E, Spielmann P, Wenger RH (2009) The PAS-domain kinase PASKIN: a new sensor in energy homeostasis. *Cell Mol Life Sci* **66**: 876-883.

14 Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**: 651-657.

15 Saleem MA, O'Hare MJ, Reiser J, Coward RJ, Inward CD, Farren T, Xing CY, Ni L, Mathieson PW, Mundel P (2002) A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* **13**: 630-638.

16 Wirthner R, Wrann S, Balamurugan K, Wenger RH, Stiehl DP (2008) Impaired DNA double-strand break repair contributes to chemoresistance in HIF-1 alpha-deficient mouse embryonic fibroblasts. *Carcinogenesis* **29**: 2306-2316.

17 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.

18 Marti HH, Katschinski DM, Wagner KF, Schaffer L, Stier B, Wenger RH (2002) Isoform-specific expression of hypoxia-inducible factor-1alpha during the late stages of mouse spermiogenesis. *Mol Endocrinol* **16**: 234-243.

19 Barth S, Edlich F, Berchner-Pfannschmidt U, Gneuss S, Jahreis G, Hasgall PA, Fandrey J, Wenger RH, Camenisch G (2009) Hypoxia-inducible factor prolyl-4-hydroxylase PHD2 protein abundance depends on integral membrane anchoring of FKBP38. *J Biol Chem* **284**: 23046-23058.

20 da Silva Xavier G, Farhan H, Kim H, Caxaria S, Johnson P, Hughes S, Bugliani M, Marselli L, Marchetti P, Birzele F, Sun G, Scharfmann R, Rutter J, Siniakowicz K, Weir G, Parker H, Reimann F, Gribble FM, Rutter GA (2010) Per-arnt-sim (PAS) domain-containing protein kinase is downregulated in human islets in type 2 diabetes and regulates glucagon secretion. *Diabetologia*.

21 Eckhardt K, Troger J, Reissmann J, Katschinski DM, Wagner KF, Stengel P, Paasch U, Hunziker P, Borter E, Barth S, Schlafli P, Spielmann P, Stiehl DP, Camenisch G, Wenger RH (2007) Male germ cell expression of the PAS domain kinase PASKIN and its novel target eukaryotic translation elongation factor eEF1A1. *Cell Physiol Biochem* **20**: 227-240.

22 Russell AP, Feilchenfeldt J, Schreiber S, Praz M, Crettenand A, Gobelet C, Meier CA, Bell DR, Kralli A, Giacobino JP, Deriaz O (2003) Endurance training in humans leads to fiber type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 and peroxisome proliferator-activated receptor-alpha in skeletal muscle. *Diabetes* **52**: 2874-2881.

23 Endlich N, Endlich K (2006) Stretch, tension and adhesion - adaptive mechanisms of the actin cytoskeleton in podocytes. *Eur J Cell Biol* **85**: 229-234.

24 Liu G, Grant WM, Persky D, Latham VM, Jr., Singer RH, Condeelis J (2002) Interactions of elongation factor 1alpha with F-actin and beta-actin mRNA: implications for anchoring mRNA in cell protrusions. *Mol Biol Cell* **13**: 579-592.

25 Kim S, Coulombe PA (2010) Emerging role for the cytoskeleton as an organizer and regulator of translation. *Nat Rev Mol Cell Biol* **11**: 75-81.

26 Waselle L, Gerona RR, Vitale N, Martin TF, Bader MF, Regazzi R (2005) Role of phosphoinositide signaling in the control of insulin exocytosis. *Mol Endocrinol* **19**: 3097-3106.

27 Tomas A, Yermen B, Regazzi R, Pessin JE, Halban PA (2010) Regulation of insulin secretion by phosphatidylinositol-4,5-bisphosphate. *Traffic* **11**: 123-137.

28 Yu M, Kasai K, Nagashima K, Torii S, Yokota-Hashimoto H, Okamoto K, Takeuchi T, Gomi H, Izumi T (2007) Exophilin4/Slp2-a targets glucagon granules to the plasma membrane through unique Ca2+-inhibitory phospholipid-binding activity of the C2A domain. *Mol Biol Cell* **18**: 688-696.

29 Shisheva A (2008) Phosphoinositides in insulin action on GLUT4 dynamics: not just PtdIns(3,4,5)P3. *Am J Physiol Endocrinol Metab* **295**: E536-544.

30 Yip MF, Ramm G, Larance M, Hoehn KL, Wagner MC, Guilhaus M, James DE (2008) CaMKII-mediated phosphorylation of the myosin motor Myo1c is required for insulin-stimulated GLUT4 translocation in adipocytes. *Cell Metab* **8**: 384-398.

31 Bose A, Guilherme A, Robida SI, Nicoloro SM, Zhou QL, Jiang ZY, Pomerleau DP, Czech MP (2002) Glucose transporter recycling in response to insulin is facilitated by myosin Myo1c. *Nature* **420**: 821-824.

10 CONCLUSIONS

PASKIN is a PAS domain containing serine/threonine kinase. Many bacterial and archaeal PAS domains are known to bind a ligand that induces conformational changes in the sensory PAS fold acting on effector domains that transduce the signal. For PASKIN, a model was proposed, where the PAS domain represses the kinase domain which in turn is de-repressed upon binding of a putative and yet unidentified ligand (*1*). Very little is known about potential phosphorylation targets of PASKIN. First hints were derived from the yeast orthologue Psk1 that regulates glycogen storage and cell wall glucan synthesis by phosphorylating glycogen synthase and UDP-glucose pyrophosphorylase as well as targets involved in protein translation (*2*). Analogously, mammalian glycogen synthase has been identified as a target of PASKIN (*3*). Another target found was pancreatic and duodenal homeobox protein 1 (PDX-1) (*4*) an important transcription factor for the expression of insulin in pancreatic β-cells. Accordingly, it has been proposed that PASKIN is involved in transcriptional regulation and secretion of insulin in pancreatic β-cells (*5*-*6*) as well as secretion of glucagon in α-cells (*7*). Further metabolic roles have been suggested due to the fact that *Paskin*$^{-/-}$ mice could be partially protected from high fat diet induced obesity and insulin resistance (8,4).

Based on all these findings, we were interested in addressing several questions to elucidating PASKIN function:

- What is the substrate specificity of PASKIN and what are potential targets that may play a role in metabolism or protein translation?
- What could be a potential ligand for the PAS domain of PASKIN and how would such a ligand influence kinase activity?
- What could be the mechanisms leading to a metabolic phenotype in *Paskin*$^{-/-}$ knockout mice?

PASKIN substrate specificity and novel kinase targets

Very recently, the structure of the kinase domain of PASKIN has been resolved and experiments regarding substrate specificity have been performed (*9*). In this report, a peptide screening revealed preference for basic residues at positions -5 and -2 and very strong preference for an arginine at position -3 relative to a serine or threonine residue. We herein present results of a microarray screening where target peptides of various kinases were spotted on a slide and were phosphorylated by recombinant PASKIN *in vitro*. Sorting of the phosphorylated hits confirmed that PASKIN kinase target peptides displayed an arginine at position -3 and that they seemed to have a similar consensus target sequence to protein kinase A and protein kinase C, themselves sharing arginines at position -3. In refining our screening

to confirm the findings from the short peptides of the microarray, we used longer peptides with a biotin tag and phosphorylated them *in vitro* by PASKIN. In this screening, we identified and validated targets of interest: the metabolic targets 6-phosphofructokinase/fructose-2,6-bisphosphatase and phosphorylase kinase β, the mitochondrial iron transport protein frataxin, the T-cell transcription factor nuclear factor of activated T-cells c4, the PAS domain containing voltage gated potassium channel hERG and ribosomal protein S6. Since it was known from yeast, that PASKIN might be involved in the regulation of protein translation (*2*), we were particularly interested in the latter. The classical view of S6 function involved its phosphorylation in upregulating translation of a specific subset of mRNAs that display a 5'-terminal oligopyrimidine tract and therefore are called 5'-TOP mRNAs (*10*). Many 5'-TOP genes are coding for components of the translational machinery itself, such as initiation factors, elongation factors and ribosomal proteins (*10-11*). Therefore, it was thought that phospho-S6 could upregulate total protein translation. Nowadays, however, it is rather thought that S6 phosphorylation might just coincide with the activation of the mTOR pathway in response to growth stimuli rather than cause increased translation (*12*), and it has even been suggested that phosphorylated S6 might be a negative feedback and therefore inhibitory to translation (*13*). As we were able to show that S6 is a target for PASKIN *in vitro*, we wondered whether we could detect differences of phospohrylation of S6 in *Paskin*$^{-/-}$ versus *Paskin*$^{+/+}$ MEFs. Not unexpectedly, S6 was highly phosphorylated in *Paskin*$^{-/-}$ MEFs most likely by p70S6 kinases. Therefore we decided to make use of S6K1/S6K2 null MEFs (*14*) and look for differences in S6 phosphorylation upon overexpression of PASKIN. Indeed, with this approach we could confirm increased phosphorylation of S6 at S235/236. However, phosphorylation of S6 did not reach the levels of wild type MEFs and we therefore assume that upon stimulation of PASKIN by an endogenous ligand, this effect could be strengthened.

We herein also present data that the eukaryotic elongation factor 1A1 is an interaction partner of PASKIN. Furthermore we could demonstrate PASKIN-mediated phosphorylation of eEF1A1 at T432 *in vitro* and functionally confirmed that PASKIN leads to increased total translation in a cell free system (*15*). Besides a role of eEF1A1 in translation, several additional functions of this protein have been suggested (see Introduction). As we herein present immunofluorescence data showing PASKIN co-localisation with the actin cytoskeleton, observations involving eEF1A1 in the context of actin bundling and remodelling are of particular interest. Indeed, eEF1A can interact directly with filamentous actin and colocalises with a phalloidin-staining at the edges of the cell (*16*). It is hypothesized that such effects may spatially restrict the translation of mRNAs and enrich also other cytoskeleton associated components of the translational machinery (*17*). Accordingly, we could show a colocalisation of PASKIN with eEF1A in the midpiece of the sperm tail, where

cytoskeletal structures are highly abundant (*15*). Most interestingly, it has been shown that eEF1A and ribosomal protein S6 both colocalise with glycine receptors in neurons (*18*). Along with colocalisation, phosphorylation of S6 could be detected upon glycine receptor activation, mediated by a yet unidentified kinase as well as cytoskeleton associated localisation of both eEF1A1 and S6 upon synaptic activity or in aging neurons. Hence, it is tempting to speculate that PASKIN might be involved in the phosphorylation of S6 in neurons and further experiments are needed to identify a function for PASKIN in actin bundling, cytoskeletal dynamics and/or vesicular transport.

PASKIN binds to phospholipids

Since PASKIN shows similarities to PKCδ in substrate specificity and both enzymes are able to phosphorylate eEF1A1 at T431 (*19*), we were wondering whether PASKIN would require the same classical phospholipid co-activators as PKCδ. Indeed, we could show that a number of phospholipids were activating PASKIN kinase activity. Protein lipid overlay assays, however, did not confirm strong binding by most of these phospholipids, but surprisingly we identified PtdIns(3)P, PtdIns(4)P and PtdIns(5)P as strong binding partners of PASKIN. We hoped that we might have discovered the long sought-after activating ligand for the PAS domain of PASKIN, however, we found that PASKIN bound to the phosphatidylinositides through the kinase domain and not through the PAS domain. Furthermore, we tested the effect of phosphatidylinositides on PASKIN autophosphorylation and target phosphorylation *in vitro*. PtdIns(3)P, PtdIns(4)P and PtdIns(5)P increased autophosphorlylation in concentration dependent manner, whereas target phosphorylation was inhibited by all phospholipids. This suggests a multiple regulation of PASKIN activity that would not only involve PAS domain binding of an activating ligand but eventually repression of kinase activity by phospholipids.

Phosphatidylinositides usually diverge in their occurrence in different membrane structures. Whereas PtdIns(4)P is very abundant in the endoplasmic reticulum and PtdIns(3)P in the endosomal compartment, the abundance of PtdIns(5)P is very low, and little is known about actual binding domains and particular functions of this phospholipid (*20*). As we also investigated subcellular localisation of PASKIN and found localisation of PASKIN at cellular edges in HeLa cells as well as along the actin cytoskeleton in the AB-81 podocyte cell line, we speculate about a role of PASKIN in vesicular trafficking and potentially in actin cytoskeleton dynamics. To fortify these findings, cellular experiments overexpressing phospholipid binding domains could eventually displace PASKIN from its subcellular localisation and provide functional links. Potential physiologically relevant functions integrating metabolism and phospholipid binding will be discussed later in this text.

PASKIN in metabolic homeostasis

Besides functions in pancreatic α and β-cells (see Introduction), PASKIN also regulates glycogen storage (*3*). Therefore, we expected a potential metabolic phenotype in muscle or liver, the main glycogen sythesizing organs. Feeding of a high fat diet (HFD) to induce the metabolic syndrome and disregulate insulin signaling is a standard method. Observing growth upon feeding a HFD of 45% fat by calories, we herein present results that *Paskin*$^{-/-}$ mice were partially protected from HFD induced obesity, and were partially protected from development of glucose intolerance, results reported also by the group of Jared Rutter (*8*). Rutter *et al.* could also show a better glucose clearance for high fat diet fed *Paskin*$^{-/-}$ mice and better palmitate and glucose oxidation in permeabilised *Paskin*$^{-/-}$ soleus muscle. Therefore, for *Paskin*$^{-/-}$ mice, a hypermetabolic phenotype was suggested. We assumed that such a phenotype could be increased when feeding a high fat diet of 60% fat by calories. However, as presented in this thesis, we failed in conforming this finding, as the animals showed similar body weight gain. A possible explanation might be that body temperature or energy expenditure could be higher in *Paskin*$^{-/-}$ mice. Therefore we decided to perform indirect calorimetry experiments and implanted sensors for continuous measurement of body temperature into 60% high fat diet fed male mice. However, not only body weight but also body temperature and the calorimetric parameters did not differ between *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ mice and we did not observe any differences in body fat distrubution, neither. Cellular energy homeostasis is mainly regulated by AMPK, a kinase sensing the ATP/AMP ratio. To deduce a role of PASKIN as a potential counterplayer of AMPK we analysed MEFs derived from *Paskin*$^{-/-}$ and *Paskin*$^{+/+}$ embryos. Making use of a phospho-AMPK (T172) antibody, we found, that *Paskin*$^{-/-}$ MEFs displayed higher levels of activated AMPK, however, intracellular ATP concentrations remained similar. As MEFs might not represent an appropriate model for cellular energy homeostasis, we tried to confirm this finding in gastrocnemius extracts of *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice, however, we did not observe any differences, neither. Rutter *et al.* confirmed our results in soleus muscle extracts, suggesting that differences might be independent from AMPK (*8*). As they showed increased glucose oxidation in permeabilised soleus muscle, it was suggested that PASKIN regulates cellular energy homeostasis independent from AMPK. Overall, the differences reported in energy homeostasis, glucose and palmitate oxidation and eventually differing ATP levels would suggest experiments with forced running on a treadmill eventually including physical training of the mice potentially showing better exercise performance for the Paskin$^{-/-}$ genotype.

Metabolism and phospholipids, it's all about secretion...

In β-cells it has been repeatidly shown that PASKIN induces insulin expression and consequently also secretion (*5-6*), whereas recent findings propagate that in α-cells PASKIN inhibits secretion of glucagon directly without altering glucagon peptide levels (*7*). Insulin in β-cells and glucagon in α-cells are stored in granules for immediate release upon generation of action potentials by Na^+ or Ca^{2+} (*21-22*). However, besides a depolarisation-dependent trigger, a machinery for membrane docking and fusion are required to release the peptide hormones. Much more is known about the secretion of insulin granules than about glucagon secretion. Insulin secretion requires an intact pool of PtdIns(4,5)P_2. This has been demonstrated by the overexpression of pleckstrin homology domains reducing the availability of PtdIns(4,5)P_2 and by knock-downs of PtdIns(4,5)P_2 generating type II PI4-kinase β (*23*), type I PIP5-kinases α (*24*) and γ (*25*) that all reduced glucose stimulated insulin secretion. For a PIP5-kinase Iα knock-down it has been demonstrated that the F-actin cytoskeletal structure was disrupted, which was accounted for the inhibition of exocytosis. Accordingly, a protein called exophilin4 has been involved in targeting glucagon granules to the plasma membrane through its C2A domains that bind phosphatidylserine and PtdIns(4,5)P_2 (*26*). As obviously the generation of a PtdIns(4,5)P_2 pool relies on the availability of its precursors PtdIns, PtdIns(4)P and PtdIns(5)P, PASKIN might directly inhibit hormone secretion at the level of granule formation, transport or fusion. To connect granule secretion with PASKIN phospholipid binding, it would be interesting to modulate phospholipid pools by overexpression of pleckstrin homology domains and knockdowns of lipid kinases in *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ islets and microscopically follow an eventual relocalisation of PASKIN.

Given the fact that *Paskin*$^{-/-}$ mice are partially protected from high fat diet induced obesity under certain laboratory conditions but not under others, might reflect a very subtle role for PASKIN that depends on minor differences in age, diet or housing. The observation that *Paskin*$^{-/-}$ mice showed better glucose clearance than *Paskin*$^{+/+}$ animals might arise from differences in insulin stimulated glucose uptake in peripheral tissues. In skeletal muscle and adipose tissue, GLUT4 is the major glucose transporter and is stored in vesicular structures that translocate to the cell surface upon insulin stimulation (*27*). This translocation seems to depend on insulin concentration and is opposed by re-integration into sorting endosomes. It is therefore tempting to speculate that *Paskin*$^{-/-}$ mice have an improved blood glucose uptake due to either an inhibitory function of PASKIN in GLUT4 exocytosis or an active role for PASKIN in GLUT4 recycling. It is well acknowledged that upon stimulation of the insulin receptor, both PI3-kinase dependent (via PtdIns(3,4,5)P_3 generation and activation of Akt) and independent processes are required for the release of GLUT4 storage vesicles to the cell membrane. Besides the importance of PtdIns(3,4,5)P_3, however, information accumulates that

GLUT4 vesicle translocation involves also the PASKIN binding PtdIns(3)P, PtdIns(4)P and PtdIns(5)P. PtdIns(3)P and PtdIns(5)P levels are transiently increased in response to acute insulin and both phospholipids can increase GLUT4 translocation to the outer membrane (*28-30*). Interestingly, PtdIns(5)P induces a breakdown of actin stress fibers and leads to F-actin reorganisation that is thought to accelerate translocation of GLUT4 (*30*). The role of PtdIns(4)P in GLUT4 translocation is less clear. GLUT4 containing vesicles that display a PI 4-kinase activity are not reactive to insulin (*31*). However, it was shown that overexpression of PI 4-kinase IIIβ negatively affects GLUT4 translocation, supporting a role of PtdIns(4)P in early steps of vesicle formation rather than in later steps of insulin responsive secretion of vesicles (*32*). Interestingly, we observed a robust expression of PASKIN in human skeletal muscle cross sections resembling to myosin stainings, and further experiments could clarify whether cells overexpressing PASKIN might inhibit insulin stimulated GLUT4 translocation.
Conversely, we found a trend towards elevated levels of free fatty acids in the plasma of HFD fed *Paskin*$^{-/-}$ mice. As a similar mode of translocation as for GLUT4 occurs for the fatty acid transporter CD36 and FATPs (Fatty Acid Transport Proteins) (*33*), our observation might reflect a reduced uptake of fatty acids to peripheral organs, suggesting the possibility of an opposite, activating role for PASKIN in fatty acid uptake. However, it has also been shown that *Paskin*$^{-/-}$ livers have reduced CD36 mRNA levels (*8*), and since adipose tissue has not been investigated in the *Paskin*$^{-/-}$ mouse model yet, it remains a completely open question, what role PASKIN plays in whole body lipid homeostasis.

In conclusion we found that the consensus sequence for PASKIN resembles the ones from PKA and PKC, requiring an arginine residue at position -3 relative to a serine or threonine residue. In accordance with findings from yeast, we could identify novel mammalian targets of PASKIN involved in metabolism and translation and characterised the phosphorylation of eEF1A1 and S6. Furthermore, we found that PASKIN was able to bind to monophosphorylated phosphatidylinositides which inhibit target phosphorylation and might represent another layer of PASKIN regulation. Subcellularly, we found increased PASKIN localisation at the edges of HeLa cells, probably focal adhesion points, and along the actin cytoskeleton in differentiated podocytes, suggesting that PASKIN might be involved in dynamic processes of membranes such as vesicular trafficking. As we found metabolic kinase targets of PASKIN in vitro, we made use of *Paskin*$^{-/-}$ mice fed high fat diets. When fed a diet of 45% fat by calories, we observed a slight partial protection of *Paskin*$^{-/-}$ mice from detrimental effects of the metabolic syndrome, however, feeding a diet of 60% fat by calories did not increase but rather cleared away the differences between *Paskin*$^{-/-}$ and control animals. The cellular mechanistics underlying the PASKIN function as a metabolic sensor remain to be elucidated and regulation by phospholipids opens a novel perspective for future research.

References

1 Amezcua CA, Harper SM, Rutter J, Gardner KH (2002) Structure and interactions of PAS kinase N-terminal PAS domain: model for intramolecular kinase regulation. *Structure* **10**: 1349-1361.

2 Rutter J, Probst BL, McKnight SL (2002) Coordinate regulation of sugar flux and translation by PAS kinase. *Cell* **111**: 17-28.

3 Wilson WA, Skurat AV, Probst B, de Paoli-Roach A, Roach PJ, Rutter J (2005) Control of mammalian glycogen synthase by PAS kinase. *Proc Natl Acad Sci U S A* **102**: 16596-16601.

4 An R, da Silva Xavier G, Hao HX, Semplici F, Rutter J, Rutter GA (2006) Regulation by Per-Arnt-Sim (PAS) kinase of pancreatic duodenal homeobox-1 nuclear import in pancreatic beta-cells. *Biochem Soc Trans* **34**: 791-793.

5 Fontes G, Semache M, Hagman DK, Tremblay C, Shah R, Rhodes CJ, Rutter J, Poitout V (2009) Involvement of Per-Arnt-Sim Kinase and extracellular-regulated kinases-1/2 in palmitate inhibition of insulin gene expression in pancreatic beta-cells. *Diabetes* **58**: 2048-2058.

6 da Silva Xavier G, Rutter J, Rutter GA (2004) Involvement of Per-Arnt-Sim (PAS) kinase in the stimulation of preproinsulin and pancreatic duodenum homeobox 1 gene expression by glucose. *Proc Natl Acad Sci U S A* **101**: 8319-8324.

7 da Silva Xavier G, Farhan H, Kim H, Caxaria S, Johnson P, Hughes S, Bugliani M, Marselli L, Marchetti P, Birzele F, Sun G, Scharfmann R, Rutter J, Siniakowicz K, Weir G, Parker H, Reimann F, Gribble FM, Rutter GA Per-arnt-sim (PAS) domain-containing protein kinase is downregulated in human islets in type 2 diabetes and regulates glucagon secretion. *Diabetologia*.

8 Hao HX, Cardon CM, Swiatek W, Cooksey RC, Smith TL, Wilde J, Boudina S, Abel ED, McClain DA, Rutter J (2007) PAS kinase is required for normal cellular energy balance. *Proc Natl Acad Sci U S A* **104**: 15466-15471.

9 Kikani CK, Antonysamy SA, Bonanno JB, Romero R, Zhang FF, Russell M, Gheyi T, Iizuka M, Emtage S, Sauder JM, Turk BE, Burley SK, Rutter J (2010) Structural bases of PAS domain-regulated kinase (PASK) activation in the absence of activation loop phosphorylation. *J Biol Chem*.

10 Meyuhas O, Avni D, Shama S (1996) Translational Control of Ribosomal Protein mRNAs in Eukaryotes. In: Hershey JWB, Matthews MB, Sonenberg N (eds) Translational Control. Cold Spring Harbor Laboratory Press, pp 363-388

11 Iadevaia V, Caldarola S, Tino E, Amaldi F, Loreni F (2008) All translation elongation factors and the e, f, and h subunits of translation initiation factor 3 are encoded by 5'-terminal oligopyrimidine (TOP) mRNAs. *Rna* **14**: 1730-1736.

12 Ruvinsky I, Sharon N, Lerer T, Cohen H, Stolovich-Rain M, Nir T, Dor Y, Zisman P, Meyuhas O (2005) Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev* **19**: 2199-2211.

13 Meyuhas O (2008) Physiological roles of ribosomal protein S6: one of its kind. *Int Rev Cell Mol Biol* **268**: 1-37.

14 Pende M, Um SH, Mieulet V, Sticker M, Goss VL, Mestan J, Mueller M, Fumagalli S, Kozma SC, Thomas G (2004) S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol* **24**: 3112-3124.

15 Eckhardt K, Troger J, Reissmann J, Katschinski DM, Wagner KF, Stengel P, Paasch U, Hunziker P, Borter E, Barth S, Schlafli P, Spielmann P, Stiehl DP, Camenisch G, Wenger RH (2007) Male germ cell expression of the PAS domain kinase PASKIN and its novel target eukaryotic translation elongation factor eEF1A1. *Cell Physiol Biochem* **20**: 227-240.

16 Liu G, Grant WM, Persky D, Latham VM, Jr., Singer RH, Condeelis J (2002) Interactions of elongation factor 1alpha with F-actin and beta-actin mRNA: implications for anchoring mRNA in cell protrusions. *Mol Biol Cell* **13**: 579-592.

17 Kim S, Coulombe PA (2010) Emerging role for the cytoskeleton as an organizer and regulator of translation. *Nat Rev Mol Cell Biol* **11**: 75-81.

18 Bluem R, Schmidt E, Corvey C, Karas M, Schlicksupp A, Kirsch J, Kuhse J (2007) Components of the translational machinery are associated with juvenile glycine receptors and are redistributed to the cytoskeleton upon aging and synaptic activity. *J Biol Chem* **282**: 37783-37793.

19 Kielbassa K, Muller HJ, Meyer HE, Marks F, Gschwendt M (1995) Protein kinase C delta-specific phosphorylation of the elongation factor eEF-alpha and an eEF-1 alpha peptide at threonine 431. *J Biol Chem* **270**: 6156-6162.

20 Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**: 651-657.

21 Eliasson L, Abdulkader F, Braun M, Galvanovskis J, Hoppa MB, Rorsman P (2008) Novel aspects of the molecular mechanisms controlling insulin secretion. *J Physiol* **586**: 3313-3324.

22 Quesada I, Tuduri E, Ripoll C, Nadal A (2008) Physiology of the pancreatic alpha-cell and glucagon secretion: role in glucose homeostasis and diabetes. *J Endocrinol* **199**: 5-19.

23 Waselle L, Gerona RR, Vitale N, Martin TF, Bader MF, Regazzi R (2005) Role of phosphoinositide signaling in the control of insulin exocytosis. *Mol Endocrinol* **19**: 3097-3106.

24 Zhang J, Luo R, Wu H, Wei S, Han W, Li G (2009) Role of type Ialpha phosphatidylinositol-4-phosphate 5-kinase in insulin secretion, glucose metabolism, and membrane potential in INS-1 beta-cells. *Endocrinology* **150**: 2127-2135.

25 Tomas A, Yermen B, Regazzi R, Pessin JE, Halban PA (2010) Regulation of insulin secretion by phosphatidylinositol-4,5-bisphosphate. *Traffic* **11**: 123-137.

26 Yu M, Kasai K, Nagashima K, Torii S, Yokota-Hashimoto H, Okamoto K, Takeuchi T, Gomi H, Izumi T (2007) Exophilin4/Slp2-a targets glucagon granules to the

plasma membrane through unique Ca2+-inhibitory phospholipid-binding activity of the C2A domain. *Mol Biol Cell* **18**: 688-696.

27 Huang S, Czech MP (2007) The GLUT4 glucose transporter. *Cell Metab* **5**: 237-252.

28 Maffucci T, Brancaccio A, Piccolo E, Stein RC, Falasca M (2003) Insulin induces phosphatidylinositol-3-phosphate formation through TC10 activation. *Embo J* **22**: 4178-4189.

29 Ishiki M, Randhawa VK, Poon V, Jebailey L, Klip A (2005) Insulin regulates the membrane arrival, fusion, and C-terminal unmasking of glucose transporter-4 via distinct phosphoinositides. *J Biol Chem* **280**: 28792-28802.

30 Sbrissa D, Ikonomov OC, Strakova J, Shisheva A (2004) Role for a novel signaling intermediate, phosphatidylinositol 5-phosphate, in insulin-regulated F-actin stress fiber breakdown and GLUT4 translocation. *Endocrinology* **145**: 4853-4865.

31 Xu Z, Huang G, Kandror KV (2006) Phosphatidylinositol 4-kinase type IIalpha is targeted specifically to cellugyrin-positive glucose transporter 4 vesicles. *Mol Endocrinol* **20**: 2890-2897.

32 Mora S, Durham PL, Smith JR, Russo AF, Jeromin A, Pessin JE (2002) NCS-1 inhibits insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes through a phosphatidylinositol 4-kinase-dependent pathway. *J Biol Chem* **277**: 27494-27500.

33 Glatz JF, Luiken JJ, Bonen A (2010) Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. *Physiol Rev* **90**: 367-417.

11 CURRICULUM VITAE

PARTICULARS

FULL NAME	Philipp SCHLÄFLI
ACADEMIC DEGREE	Dipl. phil. Nat.
DATE OF BIRTH	15. July 1979
CITIZENSHIP	Switzerland
NATIVE PLACE	Albligen (BE)

EDUCATION

1995-1999	Gymnasium Interlaken (Matura Type D, Modern Languages, 1999)
1999-2004	University of Bern, Diploma in Biochemistry (2004) Diploma Thesis: ”Development of a Secretion System for Enterococci” (Supervised by Prof. Marc Solioz, Institute of Clinical Pharmacology)
2004-2005	Internship at Actelion Pharmaceuticals (Allschwil, BL) Topic: Protein purification within the Biochemistry Department of R&D
2006-2011	University of Zürich, Institute of Physiology and Zürich Center for Integrative Human Physiology Dissertation in Integrative and Molecular Medicine (2011) PhD Thesis: “The Role of PASKIN in Metabolism and Protein Translation”

12 ORIGINAL PUBLICATIONS AND OWN CONTRIBUTIONS

Review:

Schläfli, P., Borter, E., Spielmann, P. and Wenger, R.H. (2009) The PAS-domain kinase PASKIN: a new sensor in energy homeostasis. Cell. Mol. Life Sci. 66, 876-883.

Provided Data and Figures

Original Publications:

Eckhardt, K., Tröger, J., Reissmann, J., Katschinski, D. M., Wagner, K. F., Stengel, P., Paasch, U., Hunziker, P., Borter, E., Barth, S., **Schläfli, P.**, Spielmann, P., Stiehl, D.P., Camenisch, G. and Wenger, R.H. (2007) Male germ cell expression of the PAS domain kinase PASKIN and its novel target eukaryotic translation elongation factor eEF1A1. Cell. Physiol. Biochem. 20, 227 – 240.

Expressed, purified and validated all recombinant proteins used, generated T432 mutant

Schläfli, P., Tröger, J., Eckhardt, K., Borter, E., Spielmann, P. and Wenger, R.H.(2011) Substrate preference and phosphatidylinositol monophosphate inhibition of the catalytic domain of the PAS kinase PASKIN. *Manuscript submitted.*

Everything except Fig. 1A and Fig. 3

Unpublished Manuscripts:

Schläfli, P., Borter, E., Wielinga, P.Y., Hillebrand, J.J., Spielmann, P., Löwenstein, C., Alder, B., Tobler, I., Lutz, T.A. and Wenger, R.H. PASKIN protects from detrimental effects of obesity in mice.

Everything except Fig.1, Fig. 3, Fig. 5A/B and Fig. 6

Schläfli, P., Egger, S., Item, F., Spielmann, P. and Wenger, R.H. PASKIN is predominantly expressed in leukocytes and is localized along the actin cytoskeleton.

Everything

Additional Publication, not included:

Nytko, K.J., Maeda, N., **Schläfli, P**., Spielmann, P., Wenger, R.H. and Stiehl, D.P. (2011) Vitamin C is dispensable for oxygen sensing in vivo. Blood, *Manuscript in press.*

Fig. 5, Mouse handling, blood and organ sampling.

13 ACKNOWLEDGEMENTS

I would like to express my gratitude to Prof. Roland Wenger, who gave me the possibility to perform my PhD thesis in his lab. Under his supervision, I could profit from his extensive experience, and I would like to thank for his constant scientific support.

I would like to thank the members of my PhD committee, Prof. Carsten Wagner, Dr. Daniel Stiehl and Prof. Florian Lang, for their scientific inputs.

I would like to thank the ImMed program, for providing a scientific platform and excellent education. Particular thanks also to Heidi Preisig for her inexhaustible support of the PhD students and the organization of multi-faceted retreats.

This thesis would not have been possible without the help of scientific collaborators:

- Prof. Thomas Lutz, Dr. Peter Wielinga and Christian Loewenstein from the Institute of Veterinary Physiology
- Dr. Jacquelien Hillebrand from the Institute of Animal Sciences
- Prof. Carsten Wagner and Marija Mihailova from the ZIHP Core Facility for Rodent Physiology
- Prof. Clemens Cohen and Ilka Edenhofer from the Institute of Physiology
- Matthew Adjei and Sabrina Isler from the mouse facility of our institute

Very special thanks to Patrick Spielmann for his scientific and moral support, and for his helping hands whenever needed.

I would like to thank the PASKIN crew, especially Sascha Egger and Emanuela Borter for the collaborative atmosphere and the scientific and non-scientific discussions.

Thanks to my office-mates Kasia, Margot, Hasgi, Krissi, Mattia and Renato and all the rest of the Wenger-group for the nice atmosphere (despite the noodle soups…).

Special thanks also to all my friends of the institute: Soki, Nicola, Pet, Tine, Seeli, Andrea, Tina, Anne-Sophie, Mario, Yi, Susanna, Christian and Katrin for the discussions (and gossiping) and for helping out whenever materials had run out.

Finally, I am heartily thankful to my family and to Isabel for their patience, encouragement and great support in stressful times.